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<b>(21) International Application Number:</b> PCT/US92/07683 <b>(22) International Filing Date:</b> 11 September 1992 (11.09.92)  <b>(30) Priority data:</b> 759,575 13 September 1991 (13.09.91) US  <b>(71) Applicant:</b> CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).  <b>(72) Inventors:</b> WEINER, Amy, J. ; 433 Greenbrier Court, Beni- cia, CA 94510 (US). HOUGHTON, Michael ; 53 Rose- mead Court, Danville, CA 94526 (US).  <b>(74) Agents:</b> PARK, Freddie, K. et al.; Morrison & Foerster, 755 Page Mill Road, Palo Alto, CA 94304-1003 (US).		<b>(81) Designated States:</b> AU, BG, CA, CS, FI, HU, JP, PL, RO, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMMUNOREACTIVE HEPATITIS C VIRUS POLYPEPTIDE COMPOSITIONS  <b>(57) Abstract</b>  This invention relates generally to immunoreactive polypeptide compositions comprising hepatitis type C viral epitopes, methods of using the compositions in immunological applications, and materials and methods for making the compositions.		

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5      IMMUNOREACTIVE HEPATITIS C VIRUS POLYPEPTIDE COMPOSITIONSTechnical Field

This invention relates generally to immunoreactive polypeptide compositions, methods of using  
10 the compositions in immunological applications, and materials and methods for making the compositions.

Background

The hepatitis C virus has been recently  
15 identified as the major causative agent of post-transfusion Non-A, Non-B hepatitis (NANHB), as well as a significant cause of community-acquired NANBH. Materials and methods for obtaining the viral genomic sequences are known. See, e.g. PCT Publication Nos.  
20 WO89/04669, WO90/11089 & WO90/14436.

Molecular characterization of the HCV genome indicates that it is a RNA molecule of positive polarity containing approximately 10,000 nucleotides that encodes a polyprotein of about 3011 amino acids. Several lines  
25 of evidence suggest that HCV has a similar genetic organization to the viruses of the family Flaviviridae, which includes the flavi- and pestivirus. Like its pesti- and flaviviral relatives, HCV appears to encode a large polyprotein precursor from which individual viral  
30 proteins (both structural and non-structural) are processed.

RNA-containing viruses can have relatively high rates of spontaneous mutation, i.e., reportedly on the order of  $10^{-3}$  to  $10^{-4}$  per incorporated nucleotide.  
35 Therefore, since heterogeneity and fluidity of genotype

are common in RNA viruses, there may be multiple viral isolates, which may be virulent or avirulent, within the HCV species.

5 A number of different isolates of HCV have now been identified. The sequences of these isolates demonstrate the limited heterogeneity characteristic of RNA viruses.

Isolate HCV J1.1 is described in Kubo, Y. et al. (1989), Japan. Nucl. Acids Res. 17:10367-10372; 10 Takeuchi, K. et al. (1990), Gene 91:287-291; Takeuchi et al. (1990), J. Gen. Virol. 71:3027-3033; Takeuchi et al. (1990), Nucl. Acids Res. 18:4626.

The complete coding sequences plus the 5'- and 3'-terminal sequences of two independent isolates, 15 "HCV-J" and "BK", are described by Kato et al. and Takamizawa et al, respectively. (Kato et al. (1990), Proc. Natl. Acad. Sci. USA 87:9524-9528; Takamizawa et al (1991), J. Virol. 65:1105-1113.)

20 Other publications describing HCV isolates are the following;

"HCV-1": Choo et al (1990), Brit. Med. Bull. 46:423-441; Choo et al. (1991), Proc. Natl. Acad. Sci. USA 88:2451-2455; Han et al. (1991), Proc. Natl. Acad. Sci. USA 88:1711- 25 1715; European Patent Publication No. 318,216.

"HC-J1" and "HC-J4": Okamoto et al. (1991), Japan J. Exp. Med. 60:167-177.

"HCT 18", "HCT 23", "Th", "HCT 27", "EC1" and "EC10": Weiner et al. (1991), Virol. 30 180:842-848.

"Pt-1", "HCV-K1" and "HCV-K2": Enomoto et al, There are two major types of hepatitis C virus in Japan. Division of Gastroenterology, Department of Internal Medicine, Kanazawa Medical University, Japan. 35

Clones "A", "C", "D" & "E": Tsukiyama-Kohara et al., A second group of hepatitis virus, in Virus Genes.

5           A typical approach to diagnostic and vaccine strategy is to focus on conserved viral domains. This approach, however, suffers from the disadvantage of ignoring important epitopes that may lie in variable domains.

10           It is an object of this invention to provide polypeptide compositions that are immunologically cross-reactive with multiple HCV isolates, particularly with respect to heterogeneous domains of the virus.

15   Summary of the Invention

          It has been discovered that a number of important HCV epitopes vary among viral isolates, and that these epitopes can be mapped to particular domains. This discovery allows for a strategy of producing  
20 immunologically cross-reactive polypeptide compositions that focuses on variable (rather than conserved) domains.

          Accordingly, one embodiment of the present invention is an immunoreactive composition comprising polypeptides wherein the polypeptides comprise the amino  
25 acid sequence of an epitope within a first variable domain of HCV, and at least two heterogeneous amino acid sequences from the first variable domain of distinct HCV isolates are present in the composition.

          Another embodiment of the invention is an  
30 immunoreactive composition comprising a plurality of antigen sets, wherein (a) each antigen set consists of a plurality of substantially identical polypeptides comprising the amino acid sequence of an epitope within a first variable domain of an HCV isolate, and (b) the  
35 amino acid sequence of the epitope of one set is

heterogeneous with respect to the amino acid sequence of the analogous sequence of at least one other set.

Another embodiment of the invention is an immunoreactive composition comprising a plurality of polypeptides wherein each polypeptide has the formula



wherein

R and R' are amino acid sequences of about 1-2000 amino acids, and are the same or different;

10        r and r' are 0 or 1, and are the same or different;

V is an amino acid sequence comprising the sequence of an HCV variable domain, wherein the variable domain comprises at least one epitope;

15        S is an integer  $\geq 1$ , representing a selected variable domain; and

20        n is an integer  $\geq 1$ , representing a selected HCV isolate heterogeneous at a given SV with respect to at least one other isolate having a different value for n, and n being independently selected for each x;

25        x is an integer  $\geq 1$ ; and  
with the proviso that amino acid sequences are present in the composition representing a combination selected from the group consisting of (i)  $1V_1$  and  $1V_2$ , (ii)  $1V_1$  and  $2V_2$ , and (iii)  $1V_1$  and  $2V_1$ .

Yet another embodiment of the invention is a method for preparing an immunogenic pharmaceutical composition HCV comprising:

30        (a) providing an immunoreactive composition as described above;

          (b) providing a suitable excipient; and

          (c) mixing the immunoreactive composition of (a) with the excipient of (b) in a proportion that provides an immunogenic response upon administration to a  
35        mammal.

Still another embodiment of the invention is a method for producing anti-HCV antibodies comprising administering to a mammal an effective amount of an immunoreactive composition as described above.

5 Yet another embodiment of the invention is a method of detecting antibodies to HCV within a biological sample comprising:

(a) providing a biological sample suspected of containing antibodies to HCV;

10 (b) providing an immunoreactive composition described above;

(c) reacting the biological sample of (a) with the immunoreactive composition of (b) under conditions which allow the formation of antigen-antibody complexes;  
15 and

(d) detecting the formation of antigen-antibody complexes formed between the immunoreactive composition of (a) and the antibodies of the biological sample of (b), if any.

20 Another embodiment of the invention is a kit for detecting antibodies to HCV within a biological sample comprising an immunoreactive composition as described above packaged in a suitable container.

#### 25 Brief Description of the Figures

Figure 1 schematically shows the genetic organization of the HCV genome.

Figure 2 shows a comparison of the deduced amino acid sequences of the E1 protein encoded by group I  
30 and group II HCV isolates.

Figure 3 shows a comparison of the amino acid sequences of the putative E2/NS1 region of HCV isolates.

Figure 4 are graphs showing the antigenicity profiles for the amino-terminal region of the putative  
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HCV E2/NS1 protein (amino acids 384-420), and the gp 120 V3 hypervariable region of HIV-1.

Figure 5 shows a series of graphs which give the percentage probabilities that a given residue from the amino-terminal region of HCV E2/NS1 protein (amino acids 384 to 420) will be found in either alpha-helix, beta-sheet or beta-turn secondary structural motif.

Figure 6 are bar graphs showing the reactivity of antibodies in the plasma from HCV 18 (panels A-C) or Th (Panels D-f) with overlapping biotinylated 8mer peptides derived from amino acids 384 to 415 or 416 of HCV isolates HCT 18 (A,D), Th (B,E) and HCV J1 (C,F), respectively.

Figure 7 shows the deduced amino acid sequences of two regions of the E2/NS1 polypeptide, amino acids 384-414 and 547-647, given for the Q1 and Q3 isolates.

Figure 8A shows the deduced amino acid sequences of isolates HCV J1.1 and J1.2 from amino acids 384 to 647. Figure 8B shows the deduced amino acid sequences of isolates HCT27 and HCVE1 from amino acids 384 to 651.

Figure 9 shows the entire polyprotein sequence of isolate HCV-1.

## 25 Modes of Practicing the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Maniatis, Fitch & Sambrook, MOLECULAR CLONING; A LABORATORY MANUAL (2nd ed. 1989); DNA CLONING, VOLUMES I AND II (D.N Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984);



TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984);

5 the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker,

10 eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds

15 1986); IMMUNOASSAY: A PRACTICAL GUIDE (D.W. Chan ed. 1987). All patents, patent applications, and publications mentioned herein, both above and below, are incorporated by reference herein.

HCV is a new member of the Family Flaviviridae

20 which includes the pestiviruses (Hog Cholera Virus and Bovine Viral Diarrhea Virus) and the Flaviviruses, examples of which are Dengue and Yellow Fever Virus. A scheme of the genetic organization of HCV is shown in Figure 1. Similar to the flavi- and pestiviruses, HCV

25 appears to encode a basic polypeptide domain ("C") at the N-terminus of the viral polyprotein followed by two glycoprotein domains ("E1", "E2/NS1"), upstream of the nonstructural genes NS2 through NS5. The amino acid coordinates of the putative protein domains are shown in

30 Table 1.

Table 1. The Putative Protein Domains in HCV

	<u>a.a. coordinates (approximate)</u>	<u>Protein</u>
	1 - 191	C
	192 - 383	E1
5	384 - 750	E2/NS1
	751 - 1006	NS2
	1007 - 1488	NS3
	1489 - 1959	NS4
	1960 - 3011	NS5

10

As discussed above, a number of HCV isolates have been identified. Comparative sequence analysis of complete and partial HCV sequences indicates that based upon homology at the nucleotide and amino acid levels, HCV isolates can be broadly sub-divided into at least three basic groups (Table 2). See Houghton et al., (1991) Hepatology 14:381-388. However, only partial sequence is available for the isolates in group III. Therefore, when the sequences of these isolates are more defined, one or more of these isolates may deserve separation into a different group, including a potential fourth group. Table 3 shows the sequence homologies between individual viral proteins of different HCV isolates as deduced from their nucleotide sequences. It can be seen that the proteins of the same virus group exhibit greater sequence similarity than the same proteins encoded by different virus groups (Table 3). One exception to this is the nucleocapsid protein that is highly conserved among all group I and II viral isolates sequences to date. (In Table 3, the symbol N/A signifies that the sequences were not available for comparison.) For purposes of the present invention, therefore, group I isolates can be defined as those isolates having their viral proteins, particularly E1 and E2/NS1 proteins, about 90% homologous or more at the amino acid level to

35

the isolates classified as group I herein. Group II is defined in an analogous manner. Future groups can likewise be defined in terms of viral protein homology to a prototype isolate. Subgroups can also be defined by

5 homology in limited proteins, such as the E1, E2/NS1 or NS2 proteins, or by simply higher levels of homology.

Table 2. Classification of hepatitis C viral genome RNA sequences into three basic groups.

	<u>HCV I</u>	<u>HCV II</u>	<u>HCV III</u>
10	HCV-1	HCV-J1.1	Clones A,C,D&E
	HC-J1	HC-J4	HCV-K2 (a&b)
	HCT 18	HCV-J	
	HCT 23	BK	
15	Th	HCV-K1	
	HCT 27		
	EC1		
	Pt-1		

20

Table 3. Amino Acid Homologies (%) Between Viral Proteins Encoded by Different HCV Isolates

<u>HCV</u>	<u>C</u>	<u>E1</u>	<u>E2/NS1</u>	<u>NS2</u>	<u>NS3</u>	<u>NS4</u>	<u>NS5</u>
<u>Group</u>							
25	<u>I compared to</u>						
	I	98-100	94-100	N/A	N/A	N/A	99-100
	II	97-98	77-79	78-81	75-77	91-92	90-93
	III	N/A	N/A	N/A	N/A	86	76-80
							71-74
30	<u>II compared to</u>						
	II	98-100	92-100	89-100	93-100	94-100	97-100
	III	N/A	N/A	N/A	N/A	84	76
							74-75
	<u>III compared to</u>						
35	III	N/A	N/A	N/A	N/A	N/A	91-100
							89-100

It is noteworthy that the putative viral envelope proteins encoded by the E1 and E2/NS1 genes show substantial amino acid sequence variation between groups I and II. Only NS2 exhibits a greater degree of heterogeneity, while the C, NS3, NS4 and NS5 proteins all show greater sequence conservation between groups. The sequence variation observed in the putative virion envelope proteins between groups I and II reflects a characteristic segregation of amino acids between the two groups. An example of this is shown in Figure 2 where the sequence of the E1 gene product is compared between viruses of groups I and II. The E1 amino acid sequences deduced from nucleotide sequences of HCV groups II and II are shown. In the figure, the horizontal bars indicate sequence identity with HCV-1. The asterisks indicate group-specific segregation of amino acids; the group-specific residues can be clearly identified. Group I sequences are HCV-1, HCT18, HCT23, HCT27, and HC-J1. Group II sequences are HC-J4, HCV-J, HCV J1.1, and BK. Such group-specific segregation of amino acids is also present in other gene products including gp72 encoded by the E2/NS1 gene. Figure 3 shows the comparative amino acid sequence of the putative E2/NS1 region of HCV isolates which segregate as group I and group II. The latter protein also contains an N-terminal hypervariable region ("HV") of about 30 amino acids that shows large variation between nearly all isolates. See Weiner et al. (1991), supra. This region occurs between amino acids 384 to 414, using the amino acid numbering system of HCV-1.

The putative HCV envelope glycoprotein E2/NS1 may correspond to the gp53(BVDV)/gp55 (Hog Cholera Virus) envelope polypeptide of the pestiviruses and the NS1 of

the flaviviruses, both of which confer protective immunity in hosts vaccinated with these polypeptides.

Striking similarities between the hypervariable region ("HV") and HIV-1 gp120 V3 domains with respect to degree of sequence variation, the predictive effect of amino acid changes on putative antibody binding in addition to the lack of defined secondary structure suggest that the HV domain encodes neutralizing antibodies.

The immunogenicity of the domain is shown by antibody epitope mapping experiments, described in the Examples. The results of these studies suggest that in addition to the three major groups of HCV, HV specific sub-groups also exist.

Analysis of biological samples from individuals with HCV induced NANBH indicate that individuals may be carrying two or more HCV variants simultaneously. Two co-existing HV variants were found in the plasma of one individual, J1. In addition, partial sequencing of the gene of an individual with chronic NANBH, who had intermittent flares of hepatitis, revealed that the individual, Q, was infected with two HCV variants (Q1 or Q3). Each variant was associated with only one episode of the disease. An ELISA using a Q1 or Q3 specific peptide (amino acids 396-407) showed that Q developed an antibody response to the Q1 peptide but not the corresponding Q3 peptide, suggesting that Q's recrudescence of disease was due to the appearance of an HV variant. The presence of antibodies to the Q1 peptide but lack of humoral immune response to the Q3 peptide during the second episode of disease suggest that variation in the HV domain may result from the pressure of immune selection. Amino acids 396-407 appear to be subject to the greatest selective pressure in the HV domain. These findings support the thesis that high

levels of chronicity associated with the disease might be due to an inadequate immunological host response to HCV infection and/or effective viral mechanisms of immunological evasion. Moreover, they point to the E2/NS1 HV region as a genetic region involved in a viral escape mechanism and/or an inadequate immunological response mechanism(s).

As discussed above, there are several variant regions within the HCV genome. One or more of these regions are most likely involved in a viral escape mechanism and/or an inadequate immunological response mechanism. Therefore, it is desirable to include in compositions for treatment of HCV polypeptides which would induce an immunogenic response to these variants.

In that the E1 and E2/NS1 regions of the genome encode putative envelope type polypeptides, these regions would be of particular interest with respect to immunogenicity. Thus, these regions are amongst those to which it would be particularly desirable to induce and/or increase an immune response to protect an individual against HCV infection, and to aid in the prevention of chronic recurrence of the disease in infected individuals. In addition, these regions would be amongst those from which it would be desirable to detect HCV variants which are arising during the course of infection, as well as super- or co-infection by two or more variants.

The present invention describes compositions and methods for treating individuals to prevent HCV infections, and particularly chronic HCV infections. In addition, it describes compositions and methods for detecting the presence of anti-HCV antibodies in biological samples. This latter method is particularly useful in identifying anti-HCV antibodies generated in response to immunologically distinct HCV epitopes. This

method can also be used to study the evolution of multiple variants of HCV within an infected individual. In the discussion of the invention, the following definitions are applicable.

5           The term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression  
10       modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids,  
15       etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

          As used herein, A is "substantially isolated" from B when the weight of A is at least about 70%, more  
20       preferably at least about 80%, and most preferably at least about 90% of the combined weights of A and B. The polypeptide compositions of the present invention are preferably substantially free of human or other primate tissue (including blood, serum, cell lysate, cell  
25       organelles, cellular proteins, etc.) and cell culture medium.

          A "recombinant polynucleotide" intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or  
30       manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

35

A "polynucleotide" is a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including for e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer polynucleotide, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

A "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that behaves as an autonomous unit of polynucleotide



replication within a cell; i.e., capable of replication under its own control.

A "vector" is a replicon further comprising sequences providing replication and/or expression of the open reading frame.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences which govern secretion.

A "promoter" is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the normal transcription initiation site for the adjacent structural gene.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this

region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include but is not limited to mRNA, DNA (including cDNA), and recombinant polynucleotide sequences.

As used herein, "epitope" or "antigenic determinant" means an amino acid sequence that is immunoreactive. Generally an epitope consists of at least 3 to 5 amino acids, and more usually, consists of at least about 8, or even about 10 amino acids. As used herein, an epitope of a designated polypeptide denotes epitopes with the same amino acid sequence as the epitope in the designated polypeptide, and immunologic equivalents thereof.

An "antigen" is a polypeptide containing one or more epitopes.

"Immunogenic" means the ability to elicit a cellular and/or humoral immune response. An immunogenic response may be elicited by immunoreactive polypeptides alone, or may require the presence of a carrier in the presence or absence of an adjuvant.

"Immunoreactive" refers to (1) the ability to bind immunologically to an antibody and/or to a lymphocyte antigen receptor or (2) the ability to be immunogenic.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses, inter alia, polyclonal, monoclonal, and chimeric antibodies. Examples of

chimeric antibodies are discussed in U.S. Patent Nos. 4,816,397 and 4,816,567.

An "antigen set" is defined as a composition consisting of a plurality of substantially identical polypeptides, wherein the polypeptides are comprised of  
5 an amino acid sequence of one defined epitope.

"Substantially identical polypeptides" means polypeptides that are identical with the exception of variation limited to the typical range of sequence or  
10 size variation attributable to the polypeptide's method of production; e.g., recombinant expression, chemical synthesis, tissue culture, etc. This variation does not alter the desired functional property of a composition of substantially identical polypeptides; e.g., the  
15 composition behaves immunologically as a composition of identical polypeptides. The variations may be due to, for example, alterations resulting from the secretory process during transport of the polypeptide, less than 100% efficiency in chemical synthesis, etc.

20 As used herein, a "variable domain" or "VD" of a viral protein is a domain that demonstrates a consistent pattern of amino acid variation between at least two HCV isolates or subpopulations. Preferably, the domain contains at least one epitope. Variable  
25 domains can vary from isolate to isolate by as little as 1 amino acid change. These isolates can be from the same or different HCV group(s) or subgroup(s). Variable domains can be readily identified through sequence composition among isolates, and examples of these  
30 techniques are described below. For the purposes of describing the present invention, variable domains will be defined with respect to the amino acid number of the polyprotein encoded by the genome of HCV-1 as shown in Figure 9, with the initiator methionine being designated  
35 position 1. The corresponding variable domain in another

HCV isolate is determined by aligning the two isolates sequences in a manner that brings the conserved domains outside any variable domain into maximum alignment. This can be performed with any of a number of computer software packages, such as ALIGN 1.0, available from the University of Virginia, Department of Biochemistry (Attn: Dr. William R. Pearson). See Pearson et al., (1988) Proc. Natl. Acad. Sci. USA 85:2444-2448. It is to be understood that the amino acid numbers given for a particular variable domain are somewhat subjective and a matter of choice. Thus, the beginning and end of variable domains should be understood to be approximate and to include overlapping domains or subdomains, unless otherwise indicated.

15 An epitope is the "immunologic equivalent" of another epitope in a designated polypeptide when it cross-reacts with antibodies which bind immunologically to the epitope in the designated polypeptide.

20 Epitopes typically are mapped to comprise at least about five amino acids, sometimes at least about 8 amino acids, and even about 10 or more amino acids.

The amino acid sequence comprising the HCV epitope may be linked to another polypeptide (e.g., a carrier protein), either by covalent attachment or by expressing a fused polynucleotide to form a fusion protein. If desired, one may insert or attach multiple repeats of the epitope, and/or incorporate a variety of epitopes. The carrier protein may be derived from any source, but will generally be a relatively large, immunogenic protein such as BSA, KLH, or the like. If desired, one may employ a substantially full-length HCV protein as the carrier, multiplying the number of immunogenic epitopes. Alternatively, the amino acid sequence from the HCV epitope may be linked at the amino terminus and/or carboxy terminus to a non-HCV amino acid

sequence, thus the polypeptide would be a "fusion polypeptide". Analogous types of polypeptides may be constructed using epitopes from other designated viral proteins.

5           A "variant" of a designated polypeptide refers to a polypeptide in which the amino acid sequence of the designated polypeptide has been altered by the deletion, substitution, addition or rearrangement of one or more amino acids in the sequence. Methods by which variants  
10 occur (for example, by recombination) or are made (for example, by site directed mutagenesis) are known in the art.

          "Transformation" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective  
15 of the method used for the insertion, for example, direct uptake, transduction (including viral infection), f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid or viral genome, or alternatively, may  
20 be integrated into the host genome.

          An "individual" refers to a vertebrate, particularly a member of a mammalian species, and includes but is not limited to rodents (e.g., mice, rats, hamsters, guinea pigs), rabbits, goats, pigs, cattle,  
25 sheep, and primates (e.g., chimpanzees, African Green Monkeys, baboons, orangutans, and humans).

          As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of  
30 symptoms, and (iii) the substantial or complete elimination of the virus. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

          The term "effective amount" refers to an amount  
35 of epitope-bearing polypeptide sufficient to induce an

immunogenic response in the individual to which it is administered, or to otherwise detectably immunoreact in its intended system (e.g., immunoassay). Preferably, the effective amount is sufficient to effect treatment, as defined above. The exact amount necessary will vary from application. For vaccine applications or in the generation of polyclonal antiserum/antibodies, for example, the effective amount may vary depending on the species, age, and general condition of the individual, the severity of the condition being treated, the particular polypeptide selected and its mode of administration, etc. It is also believed that effective amounts will be found within a relatively large, non-critical range. An appropriate effective amount can be readily determined using only routine experimentation.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, biopsies and also samples of in vitro cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, e.g., Mab producing myeloma cells, recombinant cells, and cell components).

The immunoreactive polypeptide compositions of the present invention comprise a mixture of isolate- or group-specific epitopes from at least one HCV VD. Thus, there will be present at least two heterogeneous amino acid sequences each defining an epitope found in distinct HCV isolates located in the same or substantially same physical location in an HCV protein; i.e. each sequence maps to the same location within the HCV genome/polypeptide. Since the sequences are

heterogeneous, the location is referred to as a variable domain (VD).

To better understand the invention, first the individual amino acid sequences that make up the compositions of the invention will be explained. Then the plurality of such sequences which are found in the compositions of the present invention will be discussed.

The amino acid sequence that characterizes the polypeptides of the present invention have a basic structure as follows:

$$L_y-Z-L'_{y'} \quad (I)$$

Z represents the amino acid sequence from a region of a protein from a selected HCV isolate, where the region comprises at least one variable domain and the variable domain comprises at least one epitope. L and L' are non-HCV amino acid sequences or HCV amino acid sequences that do not contain a variable domain, and which can be the same or different. y and y' are 0 or 1 and can be the same or different. Thus, formula I represents an amino acid sequence comprising the sequence of an HCV VD, wherein the VD comprises an epitope.

As discussed above, the epitope(s) in Z will usually comprise a minimum of about 5 amino acids, more typically a minimum of about 8 amino acids, and even more typically a minimum of about 10 amino acids.

The variable domain of Z can comprise more than one epitope. The variable domain of Z is at least as big as the combined sequences of the epitopes present, thus making it typically a minimum of about 5 amino acids when a single epitope is present. Since epitopes can overlap, the minimum amino acid sequence for combined epitopes in the variable domain may be less than the sum of the individual epitopes' sequences.

Z is the amino acid sequence of an HCV isolate comprising the above-described VD. Thus, the minimum

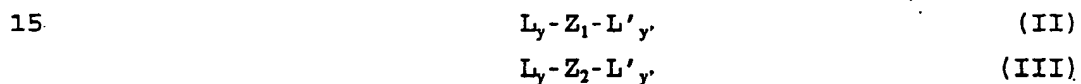
size of Z is the minimum size of the VD. Z can comprise more HCV amino acid sequence than just the VD, and can further comprise more than one VD. The maximum size of Z is not critical, but obviously cannot exceed the length of the entire HCV polyprotein. Typically, however, Z will be the sequence of an entire HCV protein (particularly E1, E2/NS1, NS2, NS3, NS4 and NS5) or, even more typically, a fragment of such an HCV protein. Thus, Z will preferably range from a minimum of about 5 amino acids (more preferably about 8 or about 10 amino acids minimum) to a maximum of about 1100 amino acids (more preferably a maximum of about 500, more preferably a maximum of about 400 or even more preferably a maximum of about 200 amino acids maximum). More usually, the polypeptide of formula I and/or Z, when prepared by, e.g., chemical synthesis, is a maximum of about 50 amino acids, more typically a maximum of about 40 amino acids, and even more typically a maximum of about 30 amino acids.

The non-HCV amino acid sequences, L and L', if present, can constitute any of a number types of such sequences. For example, L and L' can represent non-HCV sequences to which Z is fused to facilitate recombinant expression (e.g., beta-galactosidase, superoxide dismutase, invertase, alpha-factor, TPA leader, etc.); as discussed below. Alternatively, L and L' can represent epitopes of other pathogens, such as hepatitis B virus, *Bordetella pertussis*, tetanus toxoid, diphtheria, etc., to provide compositions that are immunoreactive relative to a number these other pathogens. L and L' can be amino acid sequences that facilitate attachment to solid supports during peptide synthesis, immunoassay supports, vaccine carrier proteins, etc. In fact, L and L' can even comprise one or more superfluous amino acids with no functional advantage. There is no critical maximum size



for L or L', the length being generally governed by the desired function. Typically, L and L' will each be a maximum of about 2000 amino acids, more typically a maximum of about 1000 amino acids. The majority of L and L' sequences with useful properties will be a maximum of about 500 amino acids. It is desirable, of course, to select L and L' so as to not block the immunoreactivity of Z.

The composition of polypeptides provided according to the present invention are characterized by the presence (in an effective amount for immunoreactivity) within the composition of at least two amino acid sequences defined as follows by formulas II and III, respectively:



L, L', y and y' are defined as above, as well as independently defined for each of formulas II and III. Z<sub>1</sub> and Z<sub>2</sub> are each HCV amino acid sequences as defined for Z above encompassing the same variable domain (i.e., physical location), but derived from different HCV isolates having between them at least one heterogeneous epitope in the common variable domain of Z<sub>1</sub> and Z<sub>2</sub>. As an illustrative example, an amino acid sequence according to formula II could have as Z<sub>1</sub> a fragment the hypervariable domain spanning amino acids 384-414 of isolate HCV-1 (or more particularly 396-407 or 396-408), while Z<sub>2</sub> is the analogous fragment from isolate HCV-J1.1. These two isolates are heterogeneous in this domain, the amino acid sequences of the epitopes varying significantly.

It is to be understood that the compositions of the present invention may comprise more than just two discrete amino acid sequences according to formula I, and that the Z sequences may be divided into groups encompassing different variable domains. For example, a

composition according to the present invention could comprise a group of HCV sequences (with amino acid sequences according to formula I) encompassing the hypervariable domain at amino acids 384-411 from isolates HCV-1, HCV-J1.1, HC-J1, HC-J4, etc. The composition could also comprise an additional group of HCV sequences (within amino acid sequences according to formula I) encompassing the variable domain at amino acids 215-255 also from isolates HCV-1, HCV-J1.1, HC-J1, HC-J4, etc. Within the context of the compositions of the present invention, therefore, the sequence of formula I can be further defined as follows:

$$SV_n \quad (IV)$$

V represents an amino acid sequence comprising the sequence of an HCV variable domain, wherein the variable domain comprises at least one epitope; i.e., formula I. S and n are integers of 1 or greater. S represents a particular variable domain, and n represents a particular isolate. For example, S=1 could represent the variable domain at amino acids 384-411; S=2 could represent the variable domain at amino acids 215-255; and n=1, 2, 3 and 4 could represent isolates HCV-1, HCV-J1.1, HC-J1 and HC-J4, respectively. Thus, the two groups of sequences discussed above could be represented by:

Group 1:  $1V_1, 1V_2, 1V_3$  &  $1V_4$   
Group 2:  $2V_1, 2V_2, 2V_3$  &  $2V_4$

There are at least two distinct sequences of formula IV in the compositions according to the present invention; i.e., the composition contains two different sequences according to formula IV where the values for S and or n are different. For example, at least  $1V_1$  and  $1V_2$  are present, or at least  $1V_1$  and  $2V_2$  are present, or at least  $1V_1$  and  $2V_1$  are present.

The distinct sequences falling within formula IV are present in the composition either on the same or

different polypeptide molecules. Using the minimum combination of  $1V_1$  and  $1V_2$  to illustrate, these two sequences could be present in the same polypeptide molecule (e.g.,  $1V_1-1V_2$ ) or in separate molecules. This feature of the compositions of the present invention can be described as compositions of polypeptides as follows:

$$R_r-(SV_n)_x-R'_{r'} \quad (V)$$

wherein S, V and n are as defined above; R and R' are amino acid sequences of about 1-2000 amino acids, and are the same or different; r and r' are 0 or 1, and are the same or different; x is an integer  $\geq 1$ ; n is independently selected for each x; and with the proviso that amino acid sequences are present in the composition representing a combination selected from the group consisting of (i)  $1V_1$  and  $1V_2$ , (ii)  $1V_1$  and  $2V_2$ , and (iii)  $1V_1$  and  $2V_1$ . In embodiments where the distinct sequences of formula IV are in different polypeptides, x can be 1, although it can still be  $>1$  if desired; e.g., a mixture of polypeptides  $1V_1-1V_2$  and  $1V_1-2V_2$ . When x is 1, r and r' are preferably both 0 to avoid redundancy with  $L_r$  and  $L'_{r'}$ , since V can be described by in a preferred embodiment by formula I. When x is  $>1$ , the combined lengths of R and the adjacent  $L_r$ , and of R' and the adjacent  $L'_{r'}$ , are preferably no more than the typical maximum lengths described above for L and L'.

The selection of the HCV amino acid sequences included within the distinct V sequences of the compositions will depend upon the intended application of the sequences and is within the skill of the art in view of the present disclosure. First, it should be appreciated that the HCV epitopes of concern to the present invention can be broken down into two types. The first type of epitopes are those that are "group-specific"; i.e., the corresponding epitopes in all or

substantially all isolates within an HCV isolate group are immunologically cross-reactive with each other, but not with the corresponding epitopes of substantially all the isolates of another group. Preferably, the epitopes in a group-specific class are substantially conserved within the group, but not between or among the groups. The second type of epitopes are those that are "isolate-specific"; i.e., the epitope is immunologically cross-reactive with substantially identical isolates, and is not cross-reactive with all or substantially all distinct isolates.

These group- and isolate-specific epitopes can be readily identified in view of the present disclosure. First, the sequences of several HCV isolates is compared, as described herein, and areas of sequence heterogeneity identified. The pattern of heterogeneity usually indicates group or isolate specificity. If an identified area is known to comprise one or more epitopes, then a sequence of sufficient size to include the desired epitope(s) is selected to as a variable domain that may be included in the compositions of the present invention. If the immunoreactivity of a given heterogeneous area is not known, peptides representing the sequences found in that area of the various HCV isolates can be prepared and screened. Screening can include, but is not limited too, immunoassays with various sources of anti-HCV antibody (e.g., patient serum, neutralizing Mabs, etc.) or generation of antibody and testing the ability of such antibody to neutralize virus in vitro. Alternatively, the loci of epitopes identified in a screening protocol, such as that described below, can be examined for heterogeneity among various isolates and the immunological properties of corresponding heterogeneous sequences screened.

For vaccine applications, it is believed that variable domains from the E1 and/or E2/NS1 domains will be of particular interest. In particular, an E1 variable domain within amino acids 215-255 (see Figure 2), and an  
5 E2/NS1 variable domain within amino acids 384-414 (see Figure 3), have been identified as being important immunoreactive domains. The preliminary evidence suggests that one or both of these domains may be loci of heterogeneity responsible for escape mutants, leading to  
10 chronic HCV infections. Thus, polypeptide compositions as described above where the variable domain(s) in V are one or both of these variable domains are particularly preferred. Furthermore, the polypeptide compositions of the present invention, while particularly concerned with  
15 the generally linear epitopes in the variable domains, may also include conformational epitopes. For example, the composition can be comprised of a mixture of recombinant E1 and/or E2/NS1 proteins (exhibiting the variable domains of different isolates) expressed in a  
20 recombinant system (e.g., insect or mammalian cells) that maintains conformational epitopes either inside or outside the variable domain. Alternatively, an E1 and/or E2/NS1 subunit antigen from a single isolate that maintains conformational epitopes can be combined with a  
25 polypeptide composition according to the present invention (e.g., a mixture of synthetic polypeptides or denatured recombinant polypeptides). In another preferred application for vaccines, the polypeptide compositions described herein are combined with other HCV  
30 subunit antigens, such as those described in commonly owned U.S.S.N. \_\_\_\_\_, entitled "Hepatitis C Virus Asialoglycoproteins" (Attorney Docket No. 0154.002) by Robert O. Ralston, Frank Marcus, Kent B. Thudium, Barbara Gervase, and John Hall, filed on even date herewith, and  
35 incorporated herein by reference.

For diagnostic application, it may be useful to employ the compositions of the present invention as antigens, thereby improving the ability to detect antibody to distinct HCV isolates. Typically the polypeptide mixtures can be used directly in a homogeneous or heterogeneous immunoassay format, the latter preferably comprising immobilizing the polypeptide on a solid substrate (e.g., microtiter plate wells, plastic beads, nitrocellulose, etc.). See, e.g., PCT Pub. No. WO90/11089; EPO Pub. No. 360,088; IMMUNOASSAY: A PRACTICAL GUIDE, supra. Alternatively, each substantially identical polypeptide that makes up the polypeptide composition of the present invention could be immobilized on the same support at discrete loci, thereby providing information as to which isolate or group the antibody has been generated. This may be particularly important in diagnostics if various isolates cause hepatitis, cancer or other diseases with different clinical prognoses. A preferred format is the Chiron RIBA<sup>TM</sup> strip immunoassay format, described in commonly owned U.S.S.N. 07/138,894 and U.S.S.N. 07/456,637, the disclosures of which are incorporated herein by reference.

Polypeptides useful in the manufacture of the compositions of the present invention can be made recombinantly, synthetically or in tissue culture. Recombinant polypeptides comprised of the truncated HCV sequences or full-length HCV proteins can be made up entirely of HCV sequences (one or more epitopes, either contiguous or noncontiguous), or sequences in a fusion protein. In fusion proteins, useful heterologous sequences include sequences that provide for secretion from a recombinant host, enhance the immunological reactivity of the HCV epitope(s), or facilitate the coupling of the polypeptide to a support or a vaccine

carrier. See, e.g., EPO Pub. No. 116,201; U.S. Pat. No. 4,722,840; EPO Pub. No. 259,149; U.S. Pat. No. 4,629,783, the disclosures of which are incorporated herein by reference.

5 Full length as well as polypeptides comprised of truncated HCV sequences, and mutants thereof, may be prepared by chemical synthesis. Methods of preparing polypeptides by chemical synthesis are known in the art. They may also be prepared by recombinant technology. A  
10 DNA sequence encoding HCV-1, as well as DNA sequences of variable regions from other HCV isolates have been described and/or referenced herein. The availability of these sequences permits the construction of polynucleotides encoding immunoreactive regions of HCV  
15 polypeptides.

Polynucleotides encoding the desired polypeptide comprised of one or more of the immunoreactive HCV epitope from a variable domain of HCV may be chemically synthesized or isolated, and inserted  
20 into an expression vector. The vectors may or may not contain portions of fusion sequences such as beta-Galactosidase or superoxide dismutase (SOD). Methods and vectors which are useful for the production of polypeptides which contain fusion sequences of SOD are  
25 described in European Patent Office Publication number 0196056, published October 1, 1986.

The DNA encoding the desired polypeptide, whether in fused or mature form and whether or not containing a signal sequence to permit secretion, may be  
30 ligated into expression vectors suitable for any convenient host. The hosts are then transformed with the expression vector. Both eukaryotic and prokaryotic host systems are presently used in forming recombinant polypeptides, and a summary of some of the more common  
35 control systems and host cell lines is presented infra.

The host cells are incubated under conditions which allow expression of the desired polypeptide. The polypeptide is then isolated from lysed cells or from the culture medium and purified to the extent needed for its intended use.

5           The general techniques used in extracting the HCV genome from a virus, preparing and probing DNA libraries, sequencing clones, constructing expression vectors, transforming cells, performing immunological  
10 assays such as radioimmunoassays and ELISA assays, for growing cells in culture, and the like, are known in the art. (See, e.g., the references cited in the "Background" section, above, as well as the references cited at the beginning of this ("Modes of Practicing the Invention")  
15 section above.

          Transformation of the vector containing the desired sequence into the appropriate host may be by any known method for introducing polynucleotides into a host cell, including, for example, packaging the  
20 polynucleotide in a virus and transducing the host cell with the virus, or by direct uptake of the polynucleotide. The transformation procedure used depends upon the host to be transformed. Bacterial transformation by direct uptake generally employs  
25 treatment with calcium or rubidium chloride (Cohen (1972), Proc. Natl. Acad. Sci. USA 69:2110. Yeast transformation by direct uptake may be carried out using the method of Hinnen et al. (1978), J. Adv. Enzyme Reg. 7:1929. Mammalian transformations by direct uptake  
30 may be conducted using the calcium phosphate precipitation method of Graham and Van der Eb (1978), Virology 52:546, or the various known modifications thereof. Other methods for the introduction of recombinant polynucleotides into cells, particularly into  
35 mammalian cells, which are known in the art include



dextran mediated transfection, calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the polynucleotides into nuclei.

In order to obtain expression of desired coding sequences, host cells are transformed with polynucleotides (which may be expression vectors), which are comprised of control sequences operably linked to the desired coding sequences. The control sequences are compatible with the designated host. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. Promoter sequences may be naturally occurring, for example, the  $\beta$ -lactamase (penicillinase) (Weissman (1981), "The cloning of interferon and other mistakes" in Interferon 3 (ed. I. Gresser), lactose (*lac*) (Chang et al. (1977), *Nature* 198:1056) and tryptophan (*trp*) (Goeddel et al. (1980), *Nucl. Acids Res.* 8:4057), and lambda-derived  $P_L$  promoter system and N gene ribosome binding site (Shimatake et al. (1981), *Nature* 292:128). In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one promoter may be joined with the operon sequences of another promoter, creating a synthetic hybrid promoter (e.g., the *tac* promoter, which is derived from sequences of the *trp* and *lac* promoters (De Boer et al. (1983), *Proc. Natl. Acad. Sci. USA*

80:21). The foregoing systems are particularly compatible with E. coli; if desired, other prokaryotic hosts such as strains of Bacillus or Pseudomonas may be used, with corresponding control sequences.

5 Eukaryotic hosts include yeast and mammalian cells in culture systems. Saccharomyces cerevisiae and Saccharomyces carlsbergensis are the most commonly used yeast hosts, and are convenient fungal hosts. Yeast compatible vectors generally carry markers which permit  
10 selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2 micron origin of replication (Broach et al. (1983), Meth. Enz. 101:307), the combination of CEN3  
15 and ARS1 or other means for assuring replication, such as sequences which will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors are known in the art and include promoters for the synthesis of glycolytic enzymes  
20 (Hess et al. (1968), J. Adv. Enzyme Reg. 7:149); for example, alcohol dehydrogenase (ADH) (E.P.O. Publication No. 284044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-  
25 glycerophosphate mutase, and pyruvate kinase (PyK) (E.P.O. Publication No. 329203). The yeast PHO5 gene, encoding acid phosphatase, also provides useful promoter sequences. In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For  
30 example, upstream activating sequences (UAS) of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP  
35 transcription activation region (U.S. Patent Nos.

4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the ADH2, GAL4, GAL10, or PHO5 genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (E.P.O. Publication No. 164556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase for the appropriate initiation of transcription.

Other control elements which may be included in the yeast expression vector are terminators (e.g., from GAPDH, and from the enolase gene (Holland (1981), J. Biol. Chem. 256:1385), and leader sequences. The leader sequence fragment typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene (E.P.O. Publication No. 12,873) and the  $\alpha$ -factor gene (U.S. Patent No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, also provide for secretion in yeast (E.P.O. Publication No. 60057). A preferred class of secretion leaders are those that employ a fragment of the yeast  $\alpha$ -factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of  $\alpha$ -factor fragments that can be employed include the full-length pre-pro  $\alpha$ -factor leader, as well as truncated  $\alpha$ -factor leaders (U.S. Patent Nos. 4,546,083 and 4,870,008; E.P.O. Publication No. 324274. Additional leaders employing an  $\alpha$ -factor leader fragment that provides for secretion include hybrid  $\alpha$ -factor leaders made with a pre-sequence of a first yeast, but a pro- region from a second yeast  $\alpha$ -factor. (See, e.g., P.C.T. WO 89/02463).

Expression vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for Candida albicans (Kurtz et al. (1986), Mol. Cell Biol. 6:142), Candida maltosa (Kunze et al. (1985) J. Basic Microbiol. 25:141), Hansenula polymorpha (Gleeson et al. (1986), J. Gen. Microbiol. 132:3459), Kluyveromyces fragilis (Das et al. (1984), J. Bacteriol. 158:1165), Kluyveromyces lactis (De Louvencourt et al. (1983), J. Bacteriol. 154:737), Pichia guillierimondii, (Kunze et al. (1985), supra), Pichia pastoris (Cregg et al. (1985), Mol. Cell. Biol. 5:3376; U.S. Patent Nos. 4,837,148 and 4,929,555)), Schizosaccharomyces pombe (Beach and Nurse (1981), Nature 300:706), and Yarrowia lipolytica (Davidow et al. (1985), Curr. Genet. 10:39).

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including, for example, HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, COS monkey cells, and a number of other cell lines. Suitable promoters for mammalian cells are also known in the art and include viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV) and bovine papilloma virus (BPV) (See, Sambrook (1989) for examples of suitable promoters). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art.

Vectors suitable for replication in mammalian cells are known in the art, and may include viral replicons, or sequences which ensure integration of the

appropriate sequences encoding the desired polypeptides into the host genome.

A vector which is used to express foreign DNA and which may be used in vaccine preparation is Vaccinia virus. In this case, the heterologous DNA is inserted into the Vaccinia genome. Techniques for the insertion of foreign DNA into the vaccinia virus genome are known in the art, and utilize, for example, homologous recombination. The insertion of the heterologous DNA is generally into a gene which is non-essential in nature, for example, the thymidine kinase gene (tk), which also provides a selectable marker. Plasmid vectors that greatly facilitate the construction of recombinant viruses have been described (see, for example, Mackett et al. (1984) in "DNA Cloning", Vol. II. IRL Press, p.191, Chakrabarti et al. (1985), Mol. Cell Biol. 5:3403; Moss (1987) in "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, eds., p. 10). Expression of the desired polypeptides comprised of immunoreactive regions then occurs in cells or individuals which are infected and/or immunized with the live recombinant vaccinia virus.

Other systems for expression of polypeptides include insect cells and vectors suitable for use in these cells. These systems are known in the art, and include, for example, insect expression transfer vectors derived from the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV), which is a helper-independent, viral expression vector. Expression vectors derived from this system usually use the strong viral polyhedron gene promoter to drive expression of heterologous genes. Currently the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed for improved

expression. These include, for example, pVL985 (which alters the polyhedron start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; See Luckow and Summers (1989),  
5 Virology 17:31. Good expression of nonfused foreign proteins usually requires foreign genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. The plasmid also contains the polyhedron polyadenylation  
10 signal and the ampicillin-resistance (amp) gene and origin of replication for selection and propagation in E. coli.

Methods for the introduction of heterologous DNA into the desired site in the baculovirus are known in  
15 the art. (See Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555; Ju et al. (1987), in "Gene Transfer Vectors for Mammalian Cells (Miller and Calos, eds.); Smith et al. (1983), Mol. & Cell. Biol. 3:2156; and Luckow and Summers (1989), supra). For  
20 example, the insertion can be into a gene such as the polyhedron gene, by homologous recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. The inserted sequences may be those which encode all or varying  
25 segments of the desired HCV polypeptides including at least one epitope from a variable domain.

The signals for posttranslational modifications, such as signal peptide cleavage, proteolytic cleavage, and phosphorylation, appear to be  
30 recognized by insect cells. The signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate and vertebrate cells. Examples of the signal sequences from vertebrate cells which are effective in invertebrate cells are known in  
35 the art, for example, the human interleukin 2 signal

(IL2,) which is a signal for transport out of the cell, is recognized and properly removed in insect cells.

It is often desirable that the polypeptides prepared using the above host cells and vectors be fusion polypeptides. As with non-fusion polypeptides, fusion polypeptides may remain intracellular after expression. Alternatively, fusion proteins can also be secreted from the cell into the growth medium if they are comprised of a leader sequence fragment. Preferably, there are processing sites between the leader fragment and the remainder of the foreign gene that can be cleaved either in vivo or in vitro.

In cases where the composition is to be used for treatment of HCV, it is desirable that the composition be immunogenic. In instances wherein the synthesized polypeptide is correctly configured so as to provide the correct epitope, but is too small to be immunogenic, the polypeptide may be linked to a suitable carrier. A number of techniques for obtaining such linkage are known in the art, including the formation of disulfide linkages using N-succinimidyl-3-(2-pyridylthio)propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (if the peptide lacks a sulfhydryl group, this can be provided by addition of a cysteine residue.) These reagents create a disulfide linkage between themselves and peptide cysteine residues on one protein and an amide linkage through the  $\epsilon$ -amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are known. See, for example, Immun. Rev. (1982) 62:185. Other bifunctional coupling agents for a thioether rather than a disulfide linkage. Many of these thio-ether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-

methy)cyclohexane-1-carboxylic acid, and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt. Additional methods of coupling antigens employ the rotavirus/"binding peptide" system described in EPO Publication No. 259,149. The foregoing list is not meant to be exhaustive, and modifications of the named compounds can clearly be used.

Any carrier may be used which does not itself induce the production of antibodies harmful to the host. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins; polysaccharides such as latex functionalized sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids, such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles (see infra.). Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those of skill in the art.

The immunogenicity of the epitopes of the HCV variable domains, particularly of E1 and E2/NS1, may also be enhanced by preparing them in eukaryotic systems fused with or assembled with particle-forming proteins such as, for example, that associated with hepatitis B surface antigen. See, e.g., U.S. Patent No. 4,722,840. Constructs wherein the polypeptide containing the HCV epitope from a variable domain is linked directly to the particle-forming protein coding sequences produces hybrids which are immunogenic with respect to the HCV epitope. In addition, all of the vectors prepared include epitopes specific to HBV, having various degrees of immunogenicity, such as, for example, the pre-S peptide. Thus, particles constructed from particle



forming protein which include HCV sequences are immunogenic with respect to HCV and HBV.

Hepatitis surface antigen (HBSAg) has been shown to be formed and assembled into particles in S. cerevisiae (Valenzuela et al. (1982), Nature 298:344, as well as in, for example, mammalian cells (Valenzuela et al. (1984), in "Hepatitis B", Millman I. et al., ed.). The formation of such particles has been shown to enhance the immunogenicity of the monomer subunit. The constructs may also include the immunodominant epitope of HBSAg, comprising the 55 amino acids of the presurface (pre-S) region. Neurath et al. (1984). Constructs of the pre-S-HBSAg particle expressible in yeast are disclosed in E.P.O. Publication No. 174,444; hybrids including heterologous viral sequences for yeast expression are disclosed in E.P.O. Publication No. 175,261. These constructs may also be expressed in mammalian cells such as CHO cells using an SV40-dihydrofolate reductase vector (Michelle et al. (1984)).

In addition, portions of the particle-forming protein coding sequence may be replaced with codons encoding an epitope from an HCV variable domain. In this replacement, regions which are not required to mediate the aggregation of the units to form immunogenic particles in yeast or mammals can be deleted, thus eliminating additional HBV antigenic sites from competition with the HCV epitope(s).

The preparation of vaccines which contain an immunogenic polypeptide(s) as an active ingredient(s) is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. the preparation may also be emulsified, or the polypeptide(s) encapsulated in

liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include, but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637), referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE, and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an HCV epitope from a variable domain, the antibodies resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium,

ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

In addition to the above, it is also possible to prepare live vaccines of attenuated microorganisms which express recombinant polypeptides of the HCV antigen sets. Suitable attenuated microorganisms are known in the art and include, for example, viruses (e.g., vaccinia virus) as well as bacteria.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5  $\mu$ g to 250  $\mu$ g of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of

active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each individual.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and/or reenforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the vaccine containing the antigen sets comprised of HCV polypeptides described above, may be administered in conjunction with other immunoregulatory agents, for example, immune globulins.

The compositions of the present invention can be administered to individuals to generate polyclonal antibodies (purified or isolated from serum using conventional techniques) which can then be used in a number of applications. For example, the polyclonal antibodies can be used to passively immunize an individual, or as immunochemical reagents.

In another embodiment of the invention, the above-described immunoreactive compositions comprised of a plurality of HCV antigen sets are used to detect anti-HCV antibodies within biological samples, including for example, blood or serum samples. Design of the immunoassays is subject to a great deal of variation, and a variety of these are known in the art. However, the immunoassay will use antigen sets wherein each antigen set consists of a plurality of substantially identical polypeptides comprising the amino acid sequence of an

epitope within a first variable domain of an HCV isolate, and the amino acid sequence of one set is heterogeneous with respect to the amino acid sequence of at least one other set. Protocols for the immunoassay may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the compositions of the invention containing HCV epitopes from variable domains, in suitable containers, along with the remaining reagents and materials (for example, suitable buffers, salt solutions, etc) required for the conduct of the assay, as well as a suitable set of assay instructions.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

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35

### Patient Samples and RNA Extraction

## cDNA and Polymerase Chain Reactions

30 Cloning and sequencing of HCT 18 and Th was as reported in Weiner et al. (1991), supra. Nested PCR primers used to clone the amino terminal and carboxy proximal segments of E2/NS1 in patient Q were:  
PCR. I

35 X(E2)14 GGTGCTCACTGGGGAGTCCT(1367-1386)S

X(E2)18J CATTGCAGTTCAGGGCCGTGCTA(1608-1588)A,

PCR II

X(E2)4 TCCATGGTGGGGAAGTGGGC(1406-1425)S

X(E2)19J TGCCAACTGCCATTGGTGT(1582-1562)A;

5 PCR I

X(E2)14 (above)S

J1rc12 TAACGGGCTGAGCTCGGA(2313-2296)A

PCR II

US(E2)5 CAATTGGTTCGGTTGTACC(1960-1978)S

10 J1rc13 CGTCCAGTTGCAGGCAGCTTC(2260-2240)A.

PCR primers used to clone the HCV J1 E2/NS1 gene were:

PCR I

J1(E2)14 (above)S

J1(E2)rc30" CAGGGCAGTATCTGCCACTC(2349-2330)A

15 J1IZ-2\* TGAGACGGACGTGCTGCTCCT(1960-1978)S

J1(E2)rc32" TTTGATGTACCAGGCGGCGCA(2658-2636)A

PCR II-E2384.5\*

GGATCCGCTAGCCATACCCGCGTGACGGGGGGGGTGCAA(1469-1495)S

20 DSCON1JBX\*

GGATCCTCTAGATTACTCTTCTGACCTATCCCTGTCCTCCAAGTCA  
ACA(2272-2301)A

J1IZ-1\* CAACTGGTTCGGCTGTACA(1915-1935)S

J1(E2)rc31" (2566-2546)A.

25 \*, nt sequence from Takeuchi et al., (1990) Nucl. Acids  
Res. 18:4626; ", nt sequence from Kato et al., (1989)  
Proc. Jpn. Acad. 65B:219-223. Sense (S) or antisense (A)  
PCR primers are given in the 5' to 3' orientation  
according nucleotide numbers in reference.

30

#### Synthesis of Biotinylated Peptides

The overlapping octapeptides for the  
hypervariable regions of three strains of HCV were  
synthesized on cleavable-linker, derivatized,

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polyethylene pins essentially as described by (Maeji et al., (1990) J. Immunol. Methods 134:23-33, was coupled to the N-terminus of each peptide. Finally, biotin was coupled to the N-terminus using 150  $\mu$ l of a  
5 dimethylformamide solution containing 40 mM biotin, 40 mM 1-hydroxybenzotriazole (HOBt), 40 mM benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, NOVABIOCHEM) and 60 mM N-methylmorpholine (NMM) reacting overnight at 20°C.

10 After biotinylation, the peptides were side-chain deprotected, washed and the peptide from each pin was cleaved in 200  $\mu$ l of 0.1M phosphate buffer (pH 7.2). Microtitre plates containing the cleaved peptide solutions were stored at -20°C until needed.

15

#### ELISA Testing of Biotinylated Peptides

Polystyrene plates (Nunc immuno plate maxisorb F96) were coated with streptavidin by incubating overnight at 4°C with 0.1 ml/well of a 5  $\mu$ g/ml solution  
20 of streptavidin (Sigma Cat. No. S4762) in 0.1 M carbonate buffer at pH 9.6. After removal of the streptavidin solution, the wells were washed four times with a 0.1% solution of Tween 20 in PBS. Nonspecific binding was blocked by incubating each well with 0.2 ml of 2% BSA in  
25 PBS for 1 h at 20°C. The wells were again washed four times with PBS/Tween 20. Plates were air-dried and stored at 4°C until required. The streptavidin in each well was coupled to cleaved peptides by incubation with 100  $\mu$ l of a 1:100 dilution of cleaved peptide solution  
30 with 0.1% BSA in PBS containing 0.1% sodium azide for 1 h at 20°C. After incubation, the plate was washed four times with PBS/Tween 20. Each well was incubated with 100  $\mu$ l of a suitable dilution of serum (diluted with 2% BSA in PBS containing 0.1% sodium azide) for 1 h at 20°C  
35 or overnight at 4°C followed by four washes with



PBS/Tween 20. Bound antibody was detected by reaction for 1 h at 20°C in 0.1 ml conjugate. This consisted of 0.25 ml/l (a saturating level) of horseradish peroxidase-labeled goat anti-rabbit IgG (H+L) (Kirkegaard and Perry Labs, Gaithersburg, MD) in CASS (0.1% sheep serum, 0.1% Tween 20, 0.1% sodium caseinate diluted in 0.1M PBS, pH 7.2). The wells were washed 2 times with PBS/Tween 20 followed by two washes with PBS only. The presence of enzyme was detected by reaction for 45 min at 20°C with 0.1ml of a freshly-prepared solution containing 50 mg of ammonium 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonate (ABTS, Boehringer Mannheim Cat. no. 122661) and 0.03 ml of 35% (w/w) hydrogen peroxide solution in 100 ml of 0.1 M phosphate/0.08 M citrate buffer, pH 4.0. Color development was measured in a Titertek Multiscan MC plate reader in the dual wavelength mode at 405 nm against a reference wavelength of 492 nm.

#### Computer Generated Antigenicity Profile

Antigenicity profiles for the HCV E2/NS1 protein and HIV-1 gp120 hypervariable region V3 (aa 303-338) were derived from a computer program based on the degree of sequence variability as originally proposed by Kabat [Sequences of proteins of immunological interest. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health (1983)] for the identification of the hypervariable loops of immunoglobulins multiplied by the average of the individual probability that antibody binding is retained for each possible pair-wise amino acid. Probabilities for retention of antibody binding associated with a given amino acid change were the values experimentally determined by assessing the effects on antibody binding of all possible amino acid substitutions for 103 characterized linear epitopes. Geysen et al., (1988) J.

Mol. Rec. 1:32-41. This algorithm thus weights the variability index to give more significance to amino acid changes likely to have a significant effect on antibody binding, i.e., compensates for conservative amino acid changes. Fifteen HCV sequences [HCV-1, Q3.2, HCT 23, EC10, HC-J1, HCVE1, TH, HCT 27, Q1.2, HCT18, HC-J4, HCV J1.2/HCV J1.1, HCV J , HCV BK], were used to determine the antigenicity profile for HCV. The HIV-1 V3 profile was obtained by averaging 242 individual profiles of 15 sequences selected at random from the numerically greater data base of unique HIV-1 sequences. LaRosa et al., (1990) Science 249:932-935 & Correction in Science (1991) p. 811. The amino acid sequences of some of these isolates between aa 384 and 420 are shown in Figure 3.

15

#### Computer Generated Secondary Structure Predictions

The  $\alpha$ -helix,  $\beta$ -sheet and  $\beta$ -turn secondary structure probabilities for the amino-terminal region (384-420) were determined using an algorithm, which assigns the probabilities for each of the three above secondary structural motifs to each residue. The coefficients used in the algorithm were obtained for all pair-wise combinations of residues of the structural data base. Levitt and Greer, (1977) J. Mol. Biol. 114:181-293. The prediction parameters obtained from these coefficients were fitted to the observed outcome when the algorithm was applied back on the database to obtain probabilities that a given residue would be found in one of the three defined secondary structural motifs.

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35

## Example 1

Comparison of Secondary Structure and Amino  
Acid Sequence Variation in the HCV E2/NS1 HV  
and HIV-1 gp120 Domains

The amino acid sequences from fifteen HCV and HIV-1 isolates were compared with respect to the number of positions at which amino acid sequence heterogeneities were observed in the HCV E2 HV or HIV-1 gp120 V3 domains (Figure 4, A and B, respectively). Amino acid heterogeneities occurred in 25 of 30 amino acid positions in the E2 HV region and 23 of 35 amino acid positions in the HIV-1 gp120 V3 domain. Dashes on the x-axis of Figure 4 A and B represent amino acid positions where variable amino acid residues occur and invariant amino acids are given in the single letter amino acid code. The antigenicity profiles shown in Figure 4 indicate that, similar to the V3 loop of the HIV-1 gp120 protein (Figure 4B), a block of amino acid residues in the HCV E2 (amino acids 384-414 in Figure 4A) was identified whose variation had a predicted adverse affect on antibody binding. The data in figure 4 indicate that the HCV E2 domain resembles the HIV-1 gp120 V3 domain, which is known to encode virus neutralizing epitopes, in both the degree and predicted significance of observed amino acid variation and suggests that the E2 HV domain may have a similar function as the gp120 V3 domain.

Linear epitopes are more likely associated with less structured regions of proteins, in particular, the ends of proteins or with extended surface loops. A computer analysis was used to predict the probability that an individual residue is associated with a defined secondary structural motif for 15 E2 HV amino acid sequences between residues 384 to 420. Figure 4 shows

that the region between the E2 amino-terminal residue 384 and the strongly predicted, highly conserved beta-turn (residues 415-418) is relatively unstructured as indicated by less than 50 percent probability of alpha-helix, beta-sheet or beta-turn character. Lack of strongly predictive structure in the E2 HV domain is consistent with the tolerance for extensive sequence variation found between isolates and is in contrast with highly structured regions which contribute to tertiary folding of the protein. The HCV E2 HV domain appears to be even less structured than the V3, principal neutralizing domain of HIV-1 gp120, which has been reported to contain a beta strand-type II beta turn-beta strand-alpha helix motif and may have greater structural constraints on amino acid variability than the HCV E2 HV domain. Taken together, the evidence suggests that the E2 HV domain appears to have features characteristic of protein domains which contain likely sites of linear neutralizing epitopes.

20

#### Example 2

##### Epitope Mapping of the HCV E2/NS1 HV Domain

Overlapping biotinylated 8-mer peptides corresponding to and extending past the E2/NS1 HV domain (amino acids 384 to 416) of HCT 18 (A,D), Th (B,E) and HCV J1 (C,F) were bound to plates coated with streptavidin and reacted with plasma from either HCT 18 (A-C) or Th (D-F). The results are shown in Figure 6 for HCV isolates HCT 18 (Fig. 6A and 6D), Th (Fig. 6B and 6E), and HCV J1 (Fig. 6C and 6F). HCT 18 plasma was diluted 1:200 and Th plasma was diluted 1:500. HVE-1, -2, -3, -4 and -5, represent isolate specific epitopes.

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As seen from Figure 6, HCT 18 plasma identified a linear epitope (<sup>407</sup>PKQNV<sup>411</sup>) when tested with

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peptides derived from the HCT18 sequence (HVE-I in Figure 6A), but failed to react with peptides corresponding to the HV domain of two different strains Th and HCV J1 (Figures 6B and 6C). In contrast, Th plasma  
5 identified linear epitope HVE-IV in the HV domain of Th (<sup>409</sup>QNIQLI<sup>414</sup>, Figure 6E), and also epitopes in strain HCT 18 (<sup>399</sup>IVRFFAP<sup>405</sup>, Figure 6D) and HCV J1. Th, an IV drug user, may have been exposed to multiple strains of HCV.

Both Th and HCT 18 plasma each reacted with an  
10 epitope (amino acids 413-419) common to all three isolates (data not shown) when used in an ELISA with pin synthesized overlapping 8mer peptides from each isolate.

In order to validate antibody binding specificity, antibodies bound to biotinylated peptides  
15 containing amino acids 403-407 were eluted and used to block the reactivity of HCT 18 plasma with pins containing overlapping 8-mers for the HCT 18 HV domain. These data indicate that 1) the E2/NS1 HV domain is immunogenic, 2) there are multiple epitopes which map to  
20 this region, and 3) a subset of epitopes (HVE-1, -2, -3, -4 or -5 in Figure 6) in the HV domain are isolate specific.

### Example 3

#### Determination that Variant E2/NS1 HV Domains Can Be Associated With Flares of Hepatitis

To investigate the possibility of finding HCV variants associated with the intermittent flares of hepatitis often found in chronic HCV infections, we  
30 partially sequenced the E2/NS1 gene from a patient, Q, with chronic hepatitis during two distinct episodes of hepatitis approximately two years apart (Q1 and Q3, respectively). The second episode of hepatitis occurred  
35 1.5 years after the termination of interferon treatment.

The differences in the deduced amino acid sequence of the Q1 and Q3 E2/NS1 HV region was strikingly different only between amino acids 391-408 with seven of eight changes occurring between amino acid 398 and 407 (Figure 7). Figure 7 shows the deduced amino acid sequences of two regions of the E2/NS1 polypeptide, amino acids 384-414 and 547-647, for the Q1 and Q3 isolates. The amino acid (E) above the Q1 sequence was found in one of four Q1 clones. The boxed amino acids represent the location of the Q1 or Q3 HVE 12mer peptide. Amino acid sequence differences found between Q1 and Q3 are printed in bold type.

Only one amino acid heterogeneity was observed between amino acids 547 and 647 of the Q1 and Q3 E2/NS1 polypeptides (Figure 7).

To examine the effect of the amino acid substitutions observed in the Q1 and Q3 E2 HV domains on antibody binding, we synthesized a Q1 and Q3 specific 12-mer peptide from amino acids 396 to 407 (HVE Q1 or Q3 in Figure 7B) and separately reacted the Q1 and Q3 plasma with each peptide in an ELISA. Table 4 shows that antibodies in both the Q1 and Q3 plasma reacted with the Q1 peptide but not with the Q3 peptide. Statistical analysis (Student's Test) indicated that the binding of the Q1/Q3 plasma to the Q1 peptide was significantly above background binding of those plasma to a panel of 12 randomly chosen control peptides ( $P < 0.001$ ), while binding of either the Q1 or Q3 plasma to the Q3 peptide was not statistically significant. The data indicate that although patient Q developed antibodies to the HCV Q1 HV domain, which were still detectable two years later at the Q3 time point, no detectable humoral response had developed to the Q3 E2 HV variant which was predominant during the second episode of hepatitis.

Table 4  
Elisa Results on 12-mer Peptides

5	Plasma	TARFAGFFQSGA		TAGFVRLFETGP	
		Q1 seq		Q3 seq	
		Mean	sd	Mean	sd
	Q1	1.158	0.134	0.691	0.123
	Q3	1.022	0.123	0.693	0.036

10

Example 4

Detection of Coexisting E2/NS1 Genes With  
 Distinct E2/NS1 HV Domains in HCV Infected  
 Individuals

15 Figure 8A shows the amino acid sequences deduced from two isolates of HCV J1 (J1.1 & J1.2) which were cloned from one plasma sample of the Japanese volunteer blood donor HCV J1. Kubo et al., (1989) Nucl. Acids Res. 17:10367-10372. Of the 23 total amino acid changes between HCV J1.1 and HCV J1.2, 9 differences indicated by bold type are clustered in the 30 amino acid E2/NS1 HV domain. Five of the 9 amino acid substitutions in the E2/NS1 HV domain represent nonconservative amino acid changes. Since HCV J1 is the only group II HCV genome which has been cloned in our laboratory, it is unlikely that these differences are due to cross contamination of the HCV J1 plasma. The HCV J1.2 sequence represents a minority sequence in HCV J1's blood since only two E2/NS1 HV variant sequences were identified from 25 7 cloned sequences which originated from two independent PCR reactions.

30 Interestingly, a comparison of the HCT27 and HCV E1 isolates (Figure 8B), which were sequenced in different laboratories and derive from presumably unrelated individuals, showed that the number of amino

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acid differences in the E2/NS1 HV domain of these isolates were fewer than the number of differences observed between isolates from the same individual.

The above described results lead to the suggestion that the HCV genome is rapidly evolving in individuals and the population.

#### Example 5

#### Formulation and Preparation of Vaccine

10

#### Coupling of the Diphtheria Toxoid Carrier Protein to MCS

##### Materials Required

ethylene diamine tetra-acetic acid ( $\text{EDTA Na}_2 \cdot 2\text{H}_2\text{O}$ ) (MW 372)  
6-maleimido-caproic acid N-hydroxysuccinimide ester (MCS) (Sigma) - 95% pure  
15 sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4$ )  
nitrogen  
dimethylformamide (DMF)  
Milli Q water  
0.1 M phosphate buffer containing 5 mM EDTA, pH 6.66  
0.1 M phosphate buffer, pH 8.0  
20 0.1 M phosphate buffer, pH 7.0  
sodium succinate [ $(\text{CH}_3\text{COONa})_2 \cdot 6\text{H}_2\text{O}$ ]  
cysteine  
hydrochloric acid (2% solution)  
0.1 M sodium succinate/0.1 EDTA, pH 5.6

Purified diphtheria toxoid (Commonwealth Serum Laboratories, Victoria, Australia) was coupled to MCS  
25 according to the method described by Lee et al., (1980) Mol. Immunol. 17:749; Partis et al., (1983) Prot. Chem. 2:263; Peeters et al., (1989) J. Immunol. Methods 120:133; Jones et al., (1989) J. Immunol. Methods 123:211. 100 ml of diphtheria toxoid was passed through  
30 a G25 Sephadex column (17cm X 4 cm) to remove thiomersal.  
The toxoid was eluted with 0.1 M phosphate buffer pH 7.0  
and the protein content of the eluate was assayed using  
the BCA protein determination (Pierce). The resulting

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solution was concentrated using an Amicon ultrafiltration unit to a final concentration of 10 mg/ml.

One milliliter of the toxoid solution was dialyzed with 0.1 M phosphate buffer, pH 8.0, and then mixed with a solution of 1.5 mg MCS in 200  $\mu$ l DMF. The resulting solution was incubated at room temperature for 1 hour in the dark with occasional mixing. In order to separate the uncoupled MCS from the MCS-toxoid, the solution was passed through a Sephadex PD10 column which had been equilibrated with 0.1 M phosphate buffer, pH 6.66 and the protein fraction was collected.

The number of maleimido groups coupled per carrier molecule was determined prior to coupling of the HCV peptides thereto. Thirty milliliters of the succinate/EDTA buffer was sparged with nitrogen for 2 minutes. Five milligrams of cysteine was transferred into a 25 ml volumetric flask and dissolved in a final volume of 25 ml of the sparged buffer. Aliquots of the solutions shown in Table 5 were transferred in duplicate to 25 ml screw capped bottles. Using separate pipettes, nitrogen was bubbled into each aliquot. Each bottle was then sealed and incubated at room temperature in the dark for 40 minutes with occasional swirling.

Table 5

<u>Solution</u>	<u>Sample (ml)</u>	<u>Standard (ml)</u>	<u>Blank (ml)</u>
activated carrier	0.3	-	-
phosphate buffer	-	0.3	0.3
cysteine solution	1.0	1.0	-
succinate buffer	-	-	1.0

\* A 0.1 ml aliquot of each of the 3 solution was taken for an Ellman's determination.

Ellman's Test for the Quantitative Determination of Sulfhydryl

Materials Required

Phosphate buffer, pH 8.0

- 5 Dissolve 15.6 g  $\text{NaH}_2\text{PO}_4$  or 12.0 g  $\text{NaH}_2\text{PO}_4$  anhydrous in approximately 700 ml Milli Q water. Adjust the pH to 8.0 using 50% NaOH. Add Milli Q water for a final volume of 1000 ml and then adjust the pH if necessary.

Ellman's Reagent

- 10 Dissolve 10.0 mg of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 2.5 ml of phosphate buffer, pH 8.0

0.1 ml of Ellman's reagent was added to each of the 0.1 ml aliquots of the solutions prepared above, namely the sample, standard and bland solutions. Five milliliters of phosphate buffer, pH 8.0, was then added to each aliquot, mixed well and allowed to stand for 15 minutes. The absorbance of each aliquot was measured in a 1 cm path length cell at 412 nm.

The number of maleimido groups present on the carrier protein was determined according to the following method. A 0.01  $\mu\text{mol}$  per ml solution of -SH produces an absorbance of 0.136 in a 1 cm light path at 412 nm. The absorbance of the Standard or Sample (A) is equal to the amount of cysteine reacted with the coupled maleimido groups on the activated carrier protein. Since 1 mol of available -SH reacts with 1 mol of maleimido, the concentration in  $\mu\text{mol}$ s of the maleimido groups present in the aliquot tested is equal to  $A(0.01)/0.136 \mu\text{mol/ml}$ . The total volume of the solution was 5.2 ml. Therefore, the total number of  $\mu\text{mol}$ s present was equal to  $A(0.01)(5.2)/0.136$ . The sample solution had a total volume of 1.3 ml, of which 0.3 ml consisted of the activated carrier protein. The amount of maleimido groups present in the sample solution was calculated as  $A(0.01)(5.2)(1.3)/(0.136)(0.1)(0.3) = A(16.57) \mu\text{mol/ml}$ .

The MCS-activated carrier protein was stored at -20° C.

#### Reduction of the HCV Peptides

Prior to coupling of the HCV peptides to the  
5 MCS-activated carrier protein, the peptides were reduced  
to ensure that thiol groups present on the peptides were  
in the fully reduced -SH form.

#### Materials Required

dithiothreitol (DTT)  
10 ammonium hydrogen carbonate ( $\text{NH}_4\text{HCO}_3$ )  
methanol  
SEP-PAKs (C18 cartridge, Waters), 1 cartridge for each 8  
mg of peptide  
0.1 M ammonium hydrogen carbonate buffer  
Dissolve 7.9 g  $\text{NH}_4\text{HCO}_3$  in 1 L Milli Q  
water  
15 Buffer A, 0.1% v/v trifluoroacetic acid (TFA) in Milli Q  
water  
Buffer B, 60% v/v acetonitrile, 0.1% v/v TFA in Milli Q  
water

15 mg of each of two HCV peptides corresponding  
to amino acids 384-411 and 225-260, respectively, of the  
HCV polyprotein were added to 2.5 ml of 0.1 M ammonium  
20 hydrogen carbonate containing a 10 fold molar excess of  
DTT. The resulting solutions were mixed until the  
peptide had dissolved and were then allowed to stand for  
1 hour at room temperature. Two pairs of SEP-PAKs were  
connected in series and activated by passing  
25 approximately 20 ml of methanol and then 20 ml of Buffer  
A through each pair of SEP-PAKs. Each peptide/DTT sample  
was slowly passed through a pair of SEP-PAKs. The DTT  
was eluted with 20 ml of Buffer A. The reduced peptide  
was eluted with 7 ml of Buffer B into a pre-weighed  
30 bottle and then freeze-dried overnight. The bottles were  
then weighed to determine the amount of recovered  
peptide. The reduced peptides were then immediately  
coupled to the MCS-activated carrier protein.

Coupling HCV Peptides to MCS-Activated Carrier Protein

Approximately 100 ml of 0.1 M phosphate buffer with 5 mM EDTA, pH 6.66 was degassed under vacuum and then sparged with nitrogen for 10 minutes. Twenty  
5 milliliters of a 10 mg/ml solution of the MCS-activated carrier protein was carefully sparged with nitrogen to prevent excessive frothing. 5 mg of each of the reduced peptides were dissolved in approximately 0.2 ml of the degassed sparged phosphate/EDTA buffer, pH 6.66 and then  
10 mixed with the MCS-activated carrier protein solution. The resulting mixture was transferred into a screw capped bottle which was then filled with nitrogen and sealed. The solution was further degassed by holding the bottle in a Branson 2000<sup>®</sup> sonication bath for 2 minutes. The  
15 bottle was covered with aluminum foil and incubated overnight at room temperature with slow mixing on a shaker table.

The resultant conjugate was soluble and the uncoupled peptide was removed by passing the mixture over  
20 a Sephadex PD 10 column which had been equilibrated with the phosphate/EDTA buffer, pH 6.66. The protein fraction was collected. The amount of peptide conjugated to the carrier protein was determined by amino acid analysis.

An amino acid analysis of 150  $\mu$ l aliquots of  
25 both the conjugate and the carrier protein was performed. The average ratio of the level of amino acids contributed solely by the carrier protein was determined to calculate the amount of conjugated peptide produced. Levels of serine, threonine, tryptophan, methionine, tyrosine and  
30 cysteine were not determined as these amino acids are modified under the standard hydrolysis conditions. Typical results obtained in these calculations are presented in Table 6.

Table 6

	AMINO ACID	CARRIER ONLY	CONJUGATE
	D	212	193
5	E	194	170
	G	153	108
	R	60	56
	A	150	<b>384</b>
	P	79	<b>163</b>

For the conjugate, the values in bold type are the amino acids that were also present in the peptides.

10 For conjugates containing alanine and proline, the factor  $(193+179+180+56)/(212+194+153+60) = 0.8659$  is multiplied by the amount of the amino acid level in order to normalize the result.

#### 15 Preparation of Vaccine Composition

Injectable compositions consisting of HCV peptides conjugated to MCS-activated diphtheria toxoid carrier protein prepared as described supra and a submicron oil-in-water emulsion adjuvant as described in  
 20 PCT International Publication No. WO9014837, published December 13, 1990, which is incorporated by reference herein. In addition, injectable compositions containing a an immunostimulant, lipophilic muramyl peptide (MTP-PE, CIBA-GEIGY, Basel, Switzerland) in addition to HCV  
 25 conjugated peptides and adjuvant were prepared. The vaccine compositions were generally comprised of 50% protein and 50% adjuvant.

#### Formula for Vaccine Composition with MTP-PE

30 To prepare 10 ml of injectable vaccine composition:

2.5 ml Squalene (Sigma Chemical Co., St. Louis, Mo.)  
 0.25 ml Tween 80 (Sigma Chemical Co.)  
 0.25 ml SPAN 85 (Sigma Chemical Co.)  
 1000 µg MTP-PE  
 1000 µg HCV peptide conjugated to MCS-activated  
 35 diphtheria toxoid carrier protein

Formula for Vaccine Composition without MTP-PE

To prepare 10 ml of injectabl vaccine composition:

- 2.5 ml Squalene (Sigma Chemical Co., St. Louis, Mo.)
- 0.25 ml Tween 80 (Sigma Chemical Co.)
- 5 0.25 ml SPAN 85 (Sigma Chemical Co.)
- 1000 µg HCV peptide conjugated to MCS-activated  
diphtheria toxoid carrier protein

Example 6

10 Method for Testing Vaccine  
Preparations for Toxicity

Vaccine prepared according to the methodology  
of Example 5 was tested for toxicity in small animals.  
Fifty microgram per kilogram of vaccine was administered  
15 to guinea pigs, mice and rabbits by intraperitoneal  
injection. The vaccine was also administered by  
intraperitoneal injection to rhesus monkeys and primates.  
Half of the test population of rhesus monkeys and  
primates received 5 µg/kg doses of the vaccine, while the  
20 other half received 50 µg/kg dosages. Control animals  
employed in each of the studies were injected with a  
comparable amount of a composition consisting of the  
components of the vaccine preparation except the viral  
peptides.

25 Each of the animals was monitored for symptoms  
indicative of a response to toxic material. More  
specifically, each animal in the study was examined bi-  
weekly for symptoms including fever, lethargy, weight  
loss, changes in eating habits and for lesions, swelling  
30 or tenderness at the site of injection. Lymph nodes  
proximal to the injection site were also examined for  
swelling and/or drainage. The animals were monitored on  
a bi-weekly basis for a period of several months.

Example 7Demonstration of the Production of  
Neutralizing Antibody in Vaccinated Animals

5 Vaccine prepared according to the methodology  
of Example 5 was tested in chimpanzees in order to  
determine the effectiveness of the vaccine in eliciting  
the production of virus neutralizing antibody in  
vaccinated subjects. Chimpanzees were vaccinated with 5  
10  $\mu$ g/kg dosages of vaccine prepared according to the  
methodology of Example 5 over a six-month time period at  
intervals of 0, 1, 3 and 6 months. Control chimpanzees  
were injected with comparable amounts of a composition  
consisting of the components of the vaccine except the  
15 viral peptides. Two weeks after the last dose of vaccine  
was administered, the test and control chimpanzees were  
each challenged with a 10 CIU<sub>50</sub> (Chimpanzee Infectious  
Unit) dose of CDC/910 plasma inoculum. Commencing one  
week following the viral challenge, each of the  
20 chimpanzees was monitored for viremia on a weekly basis.

In order to detect viremia, blood samples and  
liver biopsy specimens were collected from control and  
test animals on a weekly basis for several months.  
Tissue collected by liver biopsy was examined  
25 histologically for signs of necrosis and/or inflammation.  
In addition, hepatocytes from the biopsy material were  
examined by electron microscopy for the presence of  
tubules characteristic of HCV infection. The blood  
samples were also analyzed by the ELISA assay described  
30 supra for the presence of antibodies to segments of viral  
polypeptides which were not utilized in preparing the  
vaccine. In particular, each of the blood samples was  
screened by ELISA for the presence of antibodies to NS<sub>3</sub>,  
NS<sub>4</sub>, and NS<sub>5</sub> peptides. The presence of antibodies to

these peptides in the serum of a chimpanzee was indicative of HCV infection.

The following method was employed to detect viral RNA circulating in plasma or present in liver biopsy tissue collected from the chimpanzees.

cPCR Method to Detect HCV RNA in Liver and in Serum

In the cPCR assay, putative viral RNA in the sample is reverse transcribed into cDNA with reverse transcriptase; a segment of the resulting cDNA is then amplified utilizing a modified version of the PCR technique described by Saiki et al. (1986). The primers for the cPCR technique are derived from HCV RNA, which can be identified by the family of HCV cDNAs provided herein. Amplified product corresponding to the HCV-RNA is detected utilizing a probe derived from the family of HCV cDNAs provided herein.

The cPCR/HCV assay used in these studies was performed utilizing the following methods for the preparation of RNA, the reverse transcription of the RNA into cDNA, the amplification of specific segments of the cDNA by PCR, and the analysis of the PCR products.

RNA was extracted from liver utilizing the guanidium isothiocyanate method for preparing total RNA described in Maniatis et al. (1982).

In order to isolate total RNA from plasma, the plasma was diluted five- to ten-fold with TENB (0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated in a Proteinase K/SDS solution (0.5% SDS, 1 mg/ml Proteinase K, 20 micrograms/ml Poly A carrier) for 60 to 90 minutes at 37°C. The samples were extracted once with phenol (pH 6.5), the resulting organic phase was re-extracted once with TENB containing 0.1% SDS, and the aqueous phases of both extractions were pooled and extracted twice with an equal volume of phenol/CHCl<sub>3</sub>/isoamyl alcohol [1:1(99:1)].



The resulting aqueous phases were extracted with an equal volume of  $\text{CHCl}_3$ /isoamyl alcohol (99:1) twice, and ethanol precipitated using 0.2 M sodium acetate, pH 6.5, and 2.5 volumes of 100% ethanol; precipitation was overnight at  
5     $-20^\circ\text{C}$ .

The cDNA used as a template for the PCR reaction was prepared utilizing the designated samples for preparation of the corresponding cDNAs. Each RNA sample (containing either 2 micrograms of heat denatured total  
10 chimpanzee liver RNA or RNA from 2 microliters of plasma) was incubated in a 25 microliter reaction containing 1 micromolar of each primer, 1 millimolar of each deoxyribonucleotide triphosphate (dNTP), 50 millimolar Tris-HCL, pH 8.3, 5 millimolar  $\text{MgCl}_2$ , 5 millimolar  
15 dithiothreitol (DTT), 73 millimolar KCl, 40 units of RNase inhibitor (RNASIN), and 5 units of AMV reverse transcriptase. The incubation was for 60 minutes at  $37^\circ\text{C}$ . Following cDNA synthesis, the reactions were diluted with 50 microliters of deionized water (DIW),  
20 boiled for 10 minutes, and cooled on ice.

Amplification of a segment of the HCV cDNA was performed utilizing two synthetic oligomer 16-mer primers whose sequences were derived from HCV cDNA clones 36 (anti-sense) and 37b (sense). The sequence of the primer  
25 from clone 36 was:

5' GCA TGT CAT GAT GTA T 3'.

The sequence of the primer from clone 37b was:

5' ACA ATA CGT GTG TCA C 3'.

30 The primers were used at a final concentration of 1 micromolar each. In order to amplify the segment of HCV cDNA which is flanked by the primers, the cDNA samples were incubated with 0.1 microgram of RNase A and the PCR reactants of the Perkin Elmer Cetus PCR kit (N801-0043 or  
35 N801-0055) according to the manufacturer's instructions.

The PCR reaction was performed for either 30 cycles or 60 cycles in a Perkin Elmer Cetus DNA thermal cycler. Each cycle consisted of a 1 minute denaturation step at 94°C, an annealing step of 2 minutes at 37°C, and an extension step of 3 minutes at 72°C. However, the extension step in the final cycle (30 or 60) was 7 minutes rather than 3 minutes. After amplification the samples were extracted with an equal volume of phenol: chloroform (1:1), followed by extraction with an equal volume of chloroform, and then the samples were precipitated with ethanol containing 0.2 M sodium acetate.

The cPCR products were analyzed as follows. The products were subjected to electrophoresis on 1.8% alkaline agarose gels according to Murakawa et al. (1988), and transferred onto ZETA® Probe paper (BioRad Corp.) by blotting gels overnight in 0.4 M NaOH. The blots were neutralized in 2 X SSC (1 X SSC contains 0.15 M NaCl, 0.015 M sodium citrate), prehybridized in 0.3 M NaCl, 15 mM sodium phosphate buffer, pH 6.8, 15 mM EDTA, 1.0% SDS, 0.5% nonfat milk (Carnation Co.), and 0.5 mg/ml sonicated denatured salmon sperm DNA. The blots to be analyzed for HCV cDNA fragments were hybridized to a <sup>32</sup>P-labeled probe generated by nick translation of the HCV cDNA insert sequence in clone 35, described in U.S.S.N. 07/456,637. After hybridization, the blots were washed in 0.1 X SSC (1 X SSC contains 0.15M NaCl, 0.01M Na citrate) at 65°C, dried, and autoradiographed. The expected product size is 586 nucleotides in length; products which hybridized with the probe and migrated in the gels in this size range were scored as positive for viral RNA.

As a control, cPCR primers designed to amplify alpha-1 anti-trypsin mRNA was performed to verify the presence of RNA in each sample analyzed. The coding region of the alpha-1 anti-trypsin gene is described in

Rosenberg et al. (1984). Synthetic oligomer 16-mer primers designed to amplify a 365 nucleotide fragment of the coding region of the alpha-1 antitrypsin gene were derived from nucleotides 22-37 (sense) and nucleotides 372-387 (antisense). The PCR products were detected using a  $^{32}\text{P}$  nick-translated probe which lies between, and not including, the cDNA/PCR primer sequences.

Due to the extreme sensitivity of the PCR reaction, all samples were run a minimum of three times. All false positive signals were eliminated when the following precautions were taken: 1) eliminating aerosols by using screw capped tubes with rubber O-ring seals; 2) pipetting with Ranin MICROMAN® positive displacement pipettors with disposable pistons/capillaries; and 3) selecting the oligonucleotide sequences for the cDNA and PCR primers from two non-contiguous cDNA clones.

#### Industrial Utility

The immunoreactive compositions of the invention, have utility in the preparation of materials, for example, vaccines, which in turn may be used for the treatment of individuals against HCV infections, particularly chronic HCV infections. In addition, the compositions may be used to prepare materials for the detection of multiple variants of HCV in biological samples. For example, the immunoreactive compositions of the present invention can be used to generate polyclonal antibody compositions that recognize more than one HCV isolate, or as the antigen in an anti-HCV antibody immunoassay. The latter method can be used to screen blood products for possible HCV contamination. Polyclonal antiserum or antibodies can be used to for passive immunization of an individual.

Claims

WHAT IS CLAIMED IS:

- 5           1. An immunoreactive composition comprising polypeptides wherein the polypeptides comprise the amino acid sequence of an epitope within a first variable domain of a hepatitis C virus (HCV), and at least two heterogeneous amino acid sequences from the first  
10       variable domain of distinct HCV isolates are present in the composition.
2. An immunoreactive composition according to claim 1 comprising a plurality of antigen sets, wherein  
15       (a) each antigen set consists of a plurality of substantially identical polypeptides comprising the amino acid sequence of an epitope within a first variable domain of an HCV isolate, and (b) the amino acid sequence  
20       of the epitope of one set is heterogeneous with respect to the amino acid sequence of the analogous sequence of at least one other set.
3. An immunoreactive composition according to claim 1 wherein the first heterogeneous amino acid  
25       sequence is from an HCV group I isolate and the second heterogeneous amino acid sequence is from HCV group II isolate.
4. An immunoreactive composition according to  
30       claim 1 wherein the variable domain is within the E2/NS1 protein.
5. An immunoreactive composition according to  
35       claim 4 wherein the variable domain is encoded from about

amino acid 384 to about amino acid 411 of the HCV polyprotein.

6. An immunoreactive composition according to claim 1 wherein the variable domain is within the E1 protein.

7. An immunoreactive composition according to claim 6 wherein the variable domain is encoded from about amino acid 225 to about amino acid 260 of the HCV polyprotein.

8. An immunoreactive composition according to claim 1 wherein the polypeptides further comprise the amino acid sequence of an epitope within a second variable domain of a hepatitis C virus (HCV), and at least two heterogeneous amino acid sequences from the second variable domain of distinct HCV isolates are present in the composition.

9. An immunoreactive composition according to claim 8 wherein the first variable domain is within the E2/NS1 protein and the second variable domain is within the E1 protein.

10. An immunoreactive composition according to claim 1 comprising a plurality of polypeptides wherein each polypeptide has the formula



wherein

R and R' are amino acid sequences of about 1-2000 amino acids, and are the same or different;

r and r' are 0 or 1, and are the same or different;

35

V is an amino acid sequence comprising the sequence of an HCV variable domain, wherein the variable domain comprises at least one epitope;

5 S is an integer  $\geq 1$ , representing a selected variable domain; and

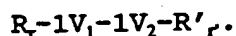
n is an integer  $\geq 1$ , representing a selected HCV isolate heterogeneous at a given SV with respect to at least one other isolate having a different value for n, and n being independently selected for each x;

10 x is an integer  $\geq 1$ ; and

with the proviso that amino acid sequences are present in the composition representing a combination selected from the group consisting of (i)  $1V_1$  and  $1V_2$ , (ii)  $1V_1$  and  $2V_2$ , and (iii)  $1V_1$  and  $2V_1$ .

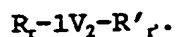
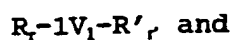
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11. The immunoreactive composition according to claim 10 wherein the polypeptide formula is



20

12. The immunoreactive composition according to claim 10 wherein the polypeptide composition comprises a mixture of polypeptides of the formulae



25

13. A method for preparing an immunogenic composition for treatment of HCV comprising:

(a) providing an immunogenic composition according to claim 1;

30

(b) providing a suitable excipient; and

(c) mixing the immunogenic composition of (a)

with the excipient of (b).

35

14. A method for producing anti-HCV antibodies comprising administering to a mammal an effective amount of an immunoreactive composition according to claim 1.

5           15. A polyclonal antibody composition made according to the method of claim 14.

16. A method of detecting antibodies to HCV within a biological sample comprising:

10           (a) providing a biological sample suspected of containing antibodies to multiple strains of HCV;

            (b) providing an immunoreactive composition according to claim 1;

            (c) reacting the biological sample of (a) with  
15 the immunoreactive composition of (b) under conditions which allow the formation of antigen-antibody complexes;  
and

            (d) detecting the formation of complexes formed between the antigen of (a) and the antibodies of  
20 the biological sample of (b), if any.

17. A kit for detecting antibodies to multiple strains of HCV within a biological sample comprising an immunoreactive composition according to claim 1 packaged  
25 in a suitable container.

18. A DNA molecule encoding a polypeptide comprising two heterogeneous amino acid sequences from the same variable domain of distinct HCV isolates.  
30

19. A host cell comprising a DNA molecule according to claim 18.

20. A host cell according to claim 19 wherein the DNA molecule comprises control sequences that are capable of causing the expression of the polypeptide.

5           21. A method of making a recombinant protein comprising growing a population of host cells according to claim 20 under conditions that provide for the expression of the polypeptide.

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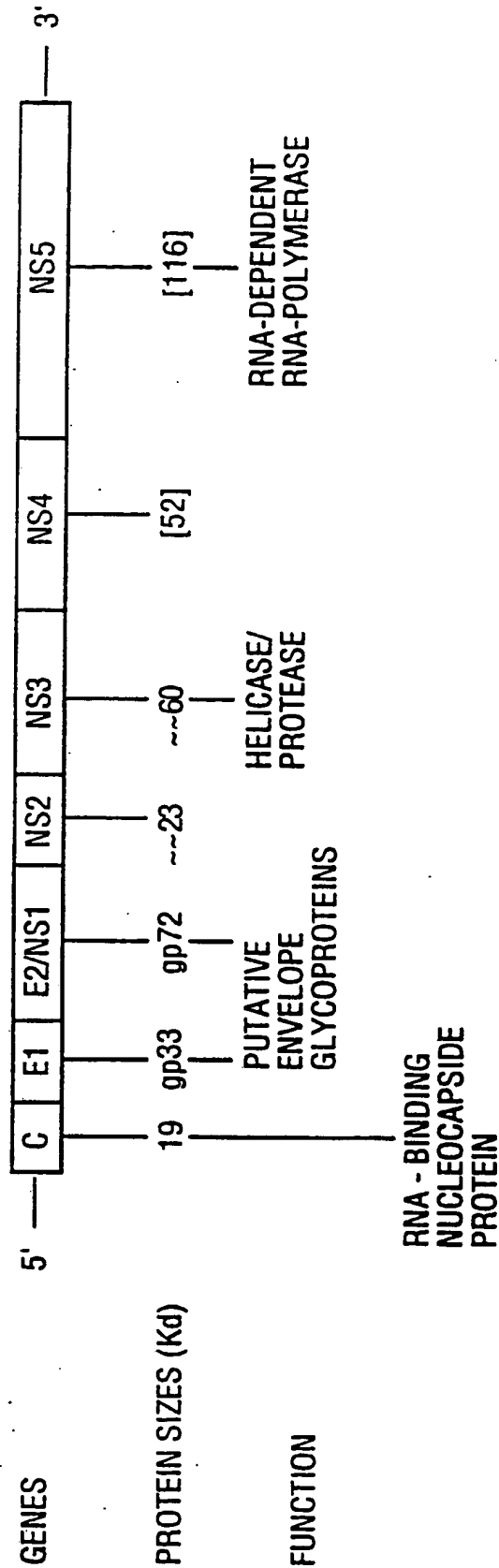


FIG. 1

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HCV-1	192	YQVRNSTGLYHVTNDCPNSSIVYEADAILHTPGCVPC
HCT18		H-----
Th		-----A-----
HCT23		-----A-----
HCT27		-----S-I-----T-T-S-----
HC-J1		-----H-----
		* * * * *
HC-J4		-E--VS-I-----S-----M-M-----
HCV-J		-E--VS-I-----S-----M-M-----
HCV J1		-E--VS-I-----S-----V-M-A-----
BK		-E-H-VS-I-----S-A-----L-M-----
HCV-1	230	VREGNASRCWVMTPTVATRDGKLPATQRRHIDLVLVGSATLCSALYVGDLCGSVFLVGQ
HCT18		-H--V-----T-----
Th		-----A--R--T-----I-----
HCT23		-----D-V-----K-----T-----I-----
HCT27		-----K--PVA-----N-----
HC-J1		-----V-----I-----
		* * * * *
HC-J4		---D-S---L--L-A-NASV-T-TI--V--A-AF-M--S--
HCV-J		---S-F---L--L-A-NSSI-T-TI--V--A-A-M--S--
HCV J1		---N-S---L--L-A-NASV-T-T--V--T-AF-M--IS--
BK		---S---L--L-A-NVTI-T-TI--V--A-AF-M--S--
HCV-1	290	LFTFSRRHWTQGCNCSIYPGHITGHRMAWDMMNWSPPTALVMAQLLRIPQAILDMIA
HCT18		-----M-----
Th		-----V-----
HCT23		-----D-----A--V-----
HCT27		-----D-----A-----
HC-J1		-----A-----
		* * **
HC-J4		---E-V-D---LS---VS---V-V--V--
HCV-J		---YE-V-D---VS---VS---V-V--V--
HCV J1		---E-V-D---VS---A--VS---VM--V--
BK		---V-L-D---VS---VS---V-V--V--

Figure 2-1



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## Comparative Amino Acid Sequence of the Putative E2/NS1 Region of HCV Isolates

HCV-1	370	KVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLIAPGAKQNVQLINTNGSWHLNSTALNC	
HCT27		---T-YT---N-AR-TQALT-FFS---	DI---I-R---
HCVE1		---L---YT---TAR-TQ-L---FSR---	DI---I-R---
H77		---R-TA-L-G-T---	I---
H90		---RS-L-IA-F-TR-P---	I-K---
Th		---T---A-GAL-IA-FNQ-R-I---	I---
HC-J1		---I-S-Q-ARAM-L---FT---	I---
HC-J4		---I-A---G-YTS-A-S-T-TLA-FS---	S-RI-V---I-R---
HCV-J		---I-M---GH---RVASSTQSL-W-SQ-PS-KI-V---	I-R---
JH-1		---I-M---GH-R---VQ-VT-TLT-FR---	S-KI-V---I-R---
BK		---I-M---GD---AQAK-TNRL-MF-S-PS-KI---	I-R---
HCV-1	430	NDSLNTGWLGLFYHHKFNSSGCPERLASCRPLTDFDQGWGPISYANGSGPDQRPYCWHY	
HCT27		-G-D-V---Y---M---A-Q---	EH---
HCVE1		-E-D-V---Y---M---A---	T-EH---
H77		-E---A-I---G---R---A---	L-E---
H90	****	-h-I---Y---	E---
Th		-E---I-Q---R---	H---
HC-J1		-H-F-A-T-R---M---IDW-A---	T-TEPDS---
HC-J4		-Q-FI-A-A-R-A---M---IDE-A---	THDMPES---
HCV-J		-Q-F-A-T-A---M---SIDK---	T-QPDNS---
JH-1		-Q-F-A-T-S---M-Q-TIDK---	T-ES-RS---
BK			
HCV-1	490	PPKPCGIVPAKSVCGPVYCFTPSPVVVGTGTTDRSGAPTYSWGENDTDVFLNNTTRPPLGNW	
HCT27		---QN---NKL---N-S-E---	
HCVE1		---QT---NKL---N-C---	
H77		---R---A---	
H90		---R---N---LI---	
Th		---N-A---	
HC-J1			
HC-J4		A-R-SQ---	
HCV-J		A-R-SQ---	F---E---LL-S---Q---
JH-1		A-RQ-SQ---	F---N-D-E---LL---H---
BK		-PQ-T---SE---	F-V---R---E---LL---Q---

Figure 3-1

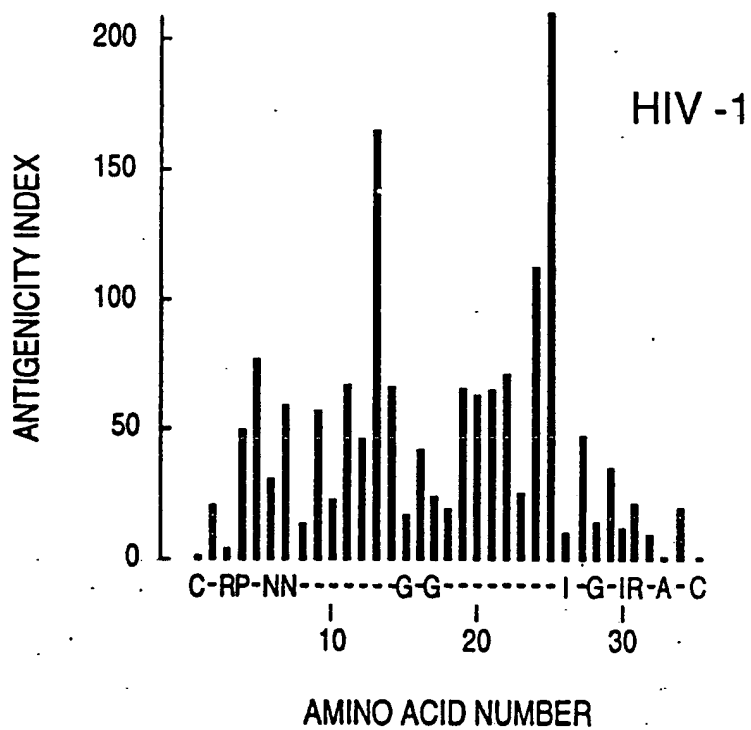
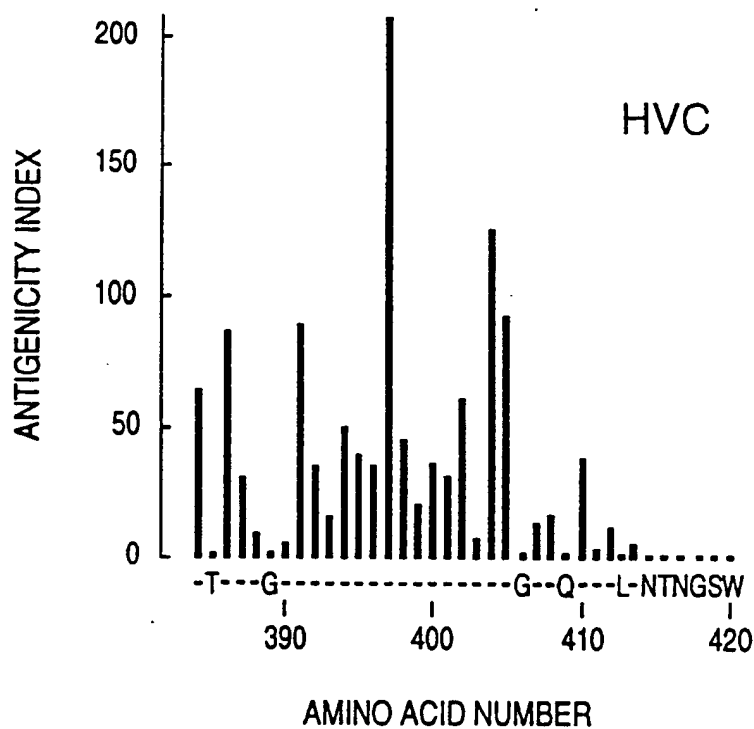
**Figure 3-2**

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HCV-1	670	TQQVLP	CSFTTLP	ALSTGL	LIHQNI	VDVQ	YLYG	VSSIA	SWAIK	WEYV	VVLL	FLLL	ADADA
HCT27		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCVE1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
H77		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
H90		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Th		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC-J1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC-J4		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-J		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
JH-1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
BK		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-1	730	RVC	SCLW	MM	LLIS	QAE	AAL	ENLV	ILNA	ASL	AGT	HGL	VSFL
HCT27		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCVE1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
H77		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
H90		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Th		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC-J1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC-J4		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-J		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-J		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
JH-1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
BK		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-1	790	FY	GM	PL	LL	LL	LL	LP	Q	A	Y	A	L
HCT27		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Th		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC-J1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC-J4		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-J		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HCV-J		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
JH-1		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
BK		-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

Figure 3-3

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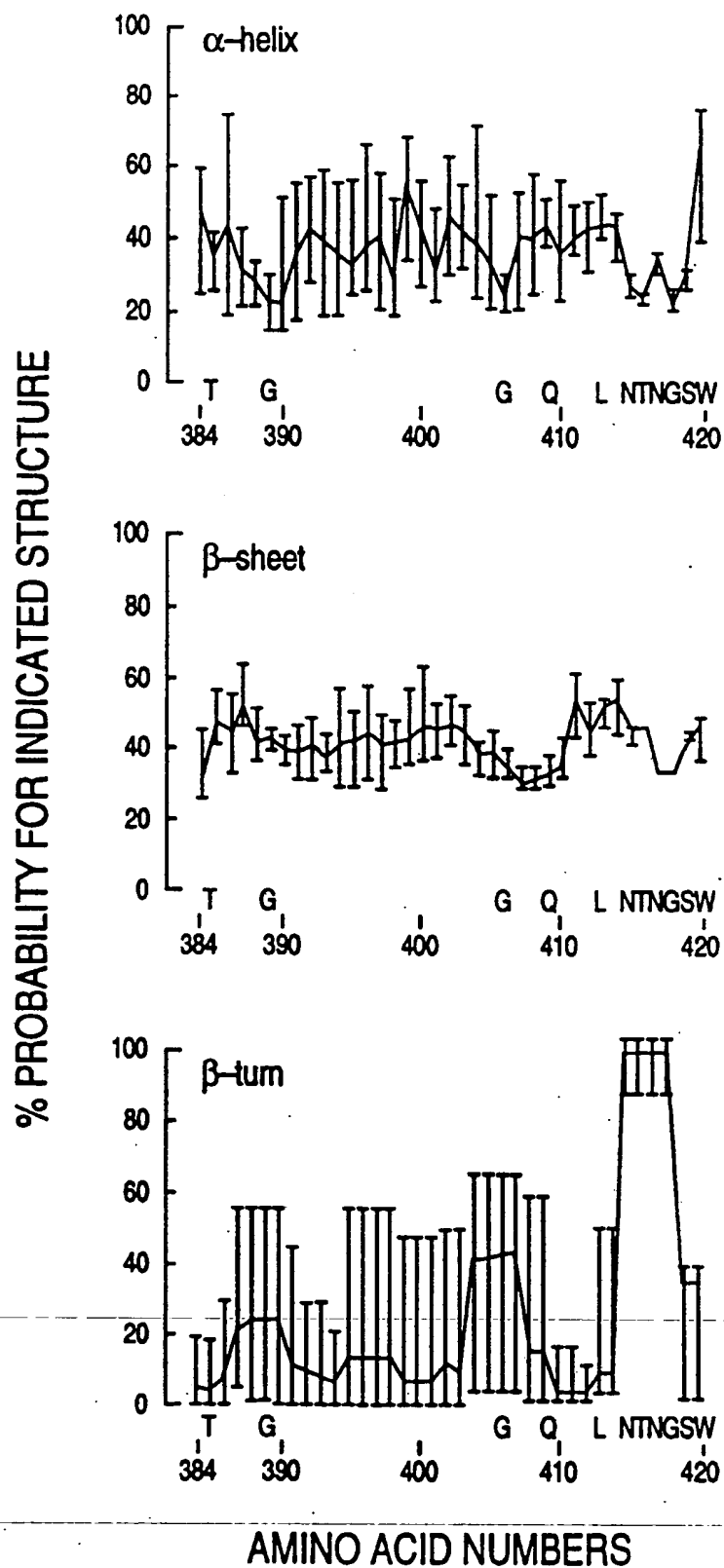
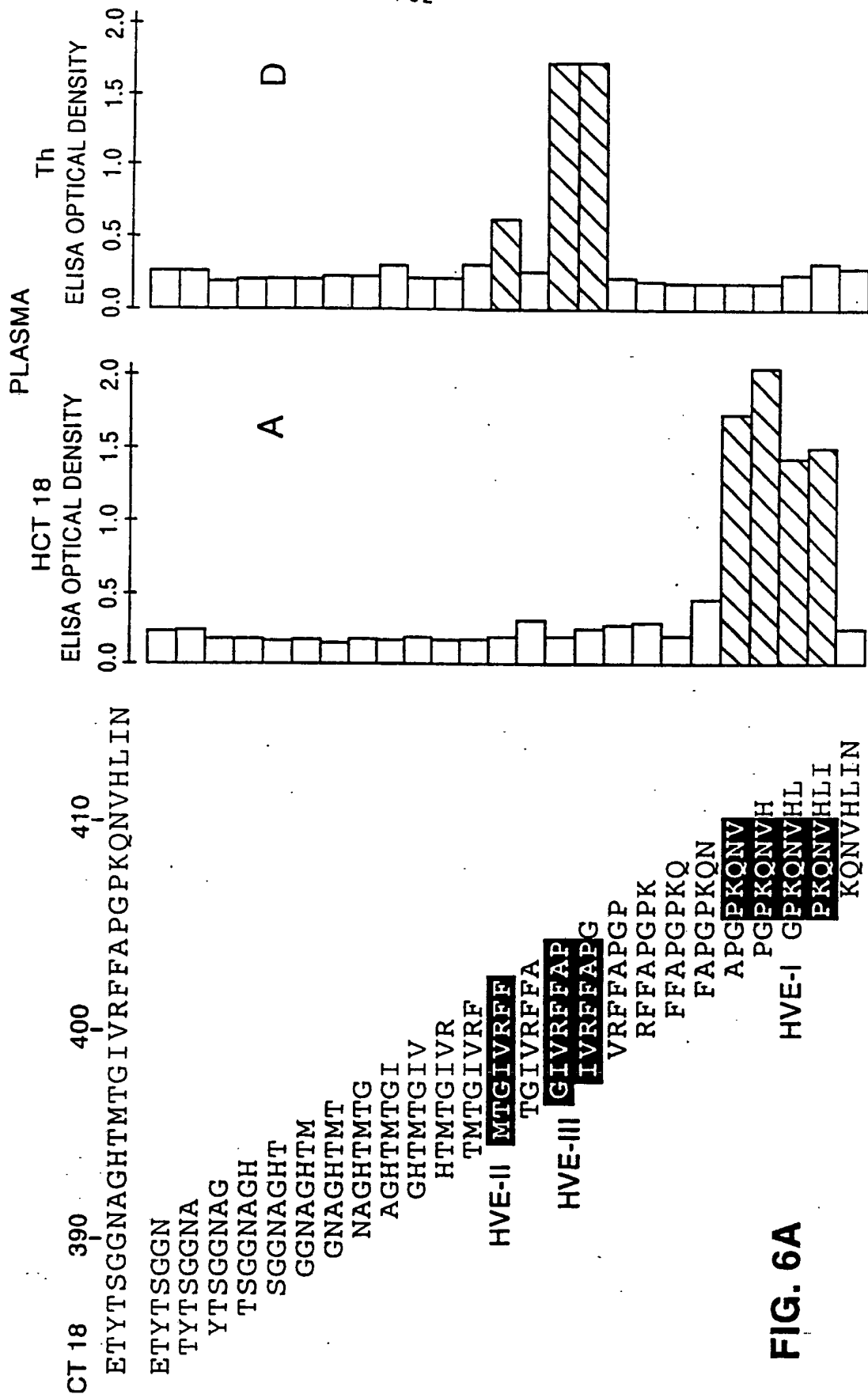


FIG. 5

SUBSTITUTE SHEET



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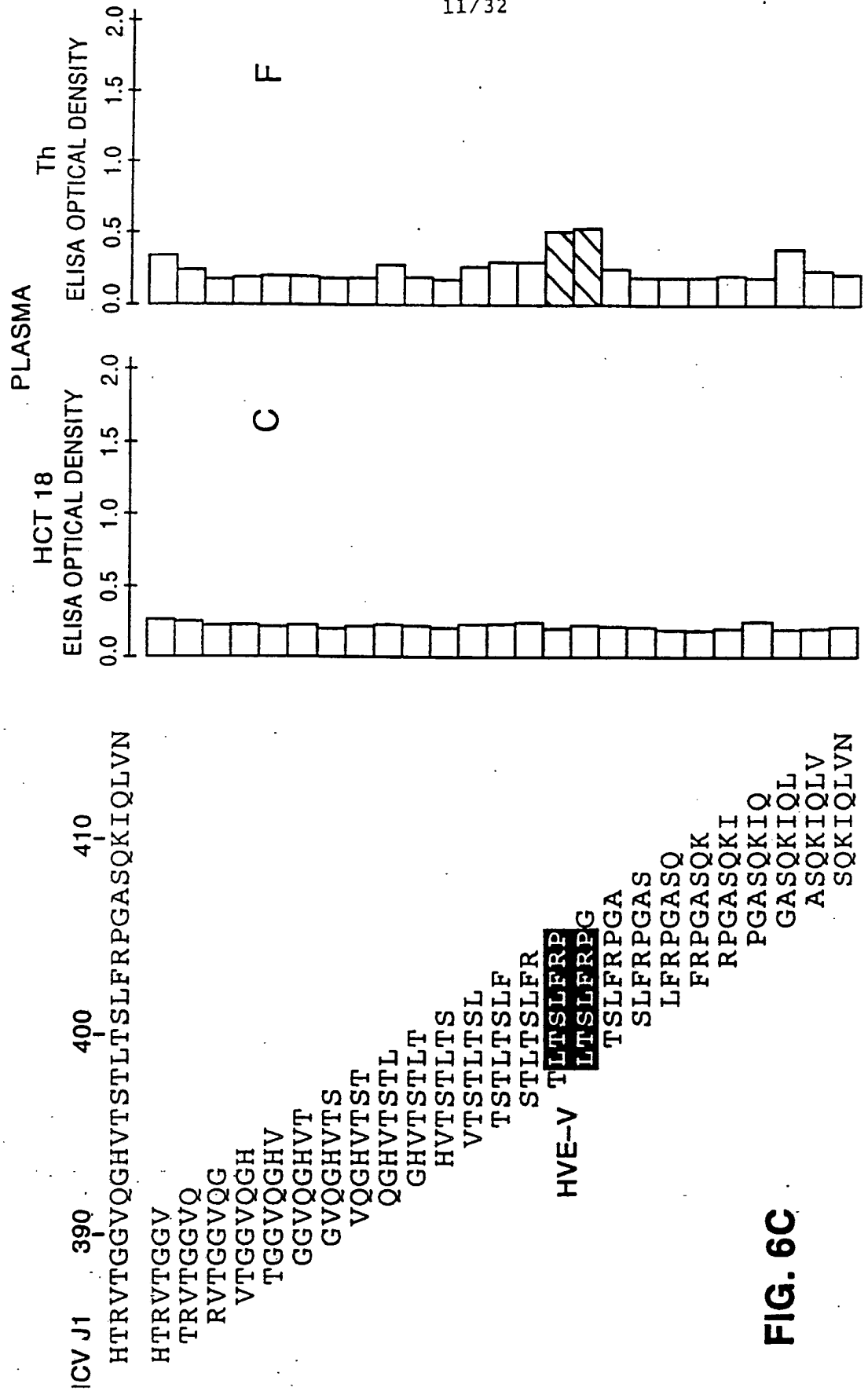


FIG. 6C

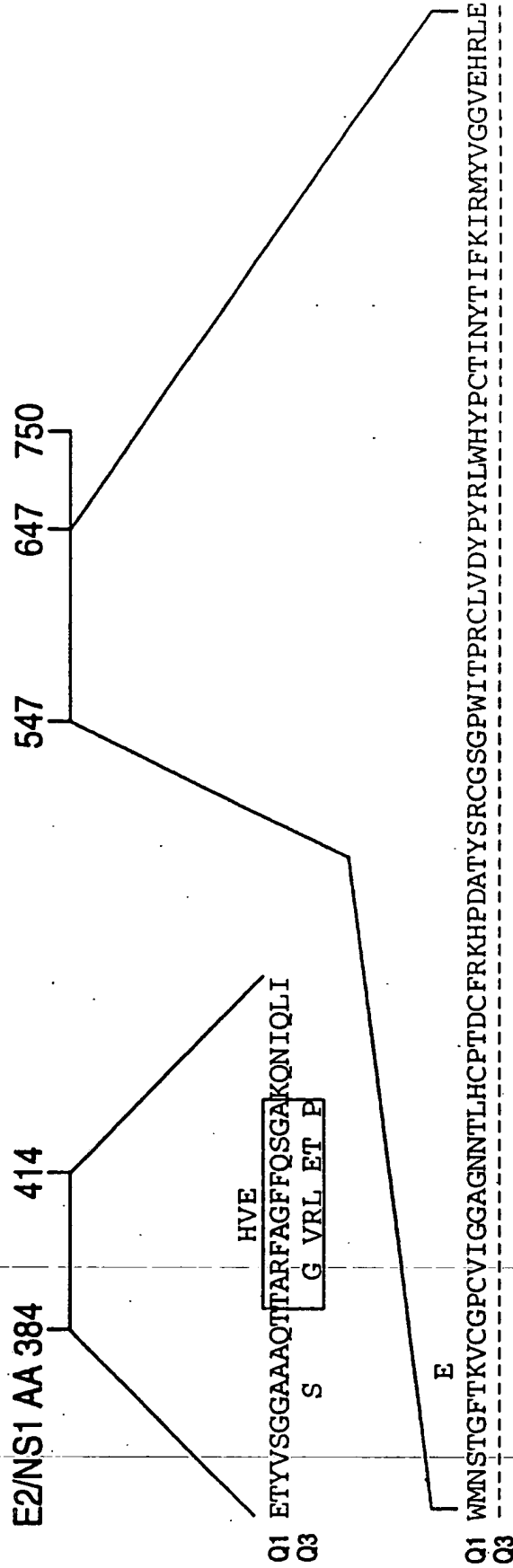


FIG. 7

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## E2 HV

HCV J1.1	384	M	HTRVTGGVQGHVTSTLTSLFRPGASQKIQLVNTNGSWHINRTALNCNDSLQGTGFLAALFY	
HCV J1.2			N-H-----GAFG-----Q-----K-----	
		R	A	
HCV J1.1	444		THKFNASGCPERMASCRSIDKFDQGWGPITYAQPDNSDQRPYCWHYAPRQCQGIVPASQVC	VG R'
HCV J1.2			--R-----T-----	
HCV J1.1	504		GPVYCFTSPSPVVVGTTDRSGAPTYNWGDNETDVLLNNTRPPHGNWEGCTWMNSTGTGTT	F V
HCV J1.2			-----	
HCV J1.1	564		CGGPPCNIGGVGNNTLTCTPTDCFRKHPDATYTKCGSGPWLTPRCLVDYPYRLWHYPCTVN	R
HCV J1.2			-----	
HCV J1.1	624		FTIFKVRMYVGGVEHRLDAACNWTRGER	K E
HCV J1.2			-----	

Figure 8-1

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## E2 HV

HCT27	384	TTYTTGGNAARTTQALTSTFFSPGAKQDIQLINTNGSWHINRTALNCNGSLDTGWVAGLFY
HCV1		E-----ST-----G-V-L-R-----E-----
HCT27	444	YHKFNSSGCPERMASCRPLADFQQGWGPISYANGSGPEHRPYCWHYPPKPGIVPAQNVC
HCV1		-----D-----T-----T-----
HCT27	504	GPVYCFTPSPVVVGTINKLGAPTYNWGSNETDVFVLNNTTRPPLGNWFGCTWMNSSGFTKV
HCV1		-----C-D-----V-----
HCT27	564	CGAPPCVIGGVGNNTLQCPTDCFRKHPDATYSRCAAGPWITPRCLVHYYPYRLWHYPCTVN
HCV1		-----A-----Y-----E-----GS-----G-----
HCT27	624	YTIVQIRMYVGVGDHRLEVACNWTGRGERCDLDDRRSELRLLLSTTQWQVLPQSFTTLP
HCV1		--LKV-----E---Q-----N-----SP-----
HCT27	684	ALTTGLIHLHQNIQVQVLYGVGSSIVSWAIKWEYVILLFLLANARICSLW
HCV1		-----D---V-----

Figure 8-2

Met Ser Thr Asn Pro Lys Pro Gln Lys Lys Asn Lys Arg Asn Thr Asn  
 1 5 10 15  
 Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gln Ile Val Gly  
 20 25 30  
 Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala  
 35 40 45  
 Thr Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro  
 50 55 60  
 Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Thr Trp Ala Gln Pro Gly  
 65 70 75 80  
 Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Cys Gly Trp Ala Gly Trp  
 85 90 95  
 Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Thr Asp Pro  
 100 105 110  
 Arg Arg Arg Ser Arg Asn Leu Gly Lys Val Ile Asp Thr Leu Thr Cys  
 115 120 125  
 Gly Phe Ala Asp Leu Met Gly Tyr Ile Pro Leu Val Gly Ala Pro Leu  
 130 135 140  
 Gly Gly Ala Ala Arg Ala Leu Ala His Gly Val Arg Val Leu Glu Asp  
 145 150 155 160  
 Gly Val Asn Tyr Ala Thr Gly Asn Leu Pro Gly Cys Ser Phe Ser Ile  
 165 170 175

FIGURE 9-1

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Phe Leu Leu Ala Leu Leu Ser Cys Leu Thr Val Pro Ala Ser Ala Tyr  
 180 185  
 Gln Val Arg Asn Ser Thr Gly Leu Tyr His Val Thr Asn Asp Cys Pro  
 195 200  
 Asn Ser Ser Ile Val Tyr Glu Ala Ala Asp Ala Ile Leu His Thr Pro  
 210 215 220  
 Gly Cys Val Pro Cys Val Arg Glu Gly Asn Ala Ser Arg Cys Trp Val  
 225 230 235 240  
 Ala Met Thr Pro Thr Val Ala Thr Arg Asp Gly Lys Leu Pro Ala Thr  
 245 250 255  
 Gln Leu Arg Arg His Ile Asp Leu Leu Val Gly Ser Ala Thr Leu Cys  
 260 265 270  
 Ser Ala Leu Tyr Val Gly Asp Leu Cys Gly Ser Val Phe Leu Val Gly  
 275 280 285  
 Gln Leu Phe Thr Phe Ser Pro Arg Arg His Trp Thr Thr Gln Gly Cys  
 290 295 300  
 Asn Cys Ser Ile Tyr Pro Gly His Ile Thr Gly His Arg Met Ala Trp  
 305 310 315 320  
 Asp Met Met Met Asn Trp Ser Pro Thr Thr Ala Leu Val Met Ala Gln  
 325 330 335  
 Leu Leu Arg Ile Pro Gln Ala Ile Leu Asp Met Ile Ala Gly Ala His  
 340 345 350

FIGURE 9-2



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Trp Gly Val Leu Ala Gly Ile Ala Tyr Phe Ser Met Val Gly Asn Trp  
 355 360 365  
 Ala Lys Val Leu Val Leu Leu Leu Phe Ala Gly Val Asp Ala Glu  
 370 375 380  
 Thr His Val Thr Gly Gly Ser Ala Gly His Thr Val Ser Gly Phe Val  
 385 390 395 400  
 Ser Leu Leu Ala Pro Gly Ala Lys Gln Asn Val Gln Leu Ile Asn Thr  
 405 410 415  
 Asn Gly Ser Trp His Leu Asn Ser Thr Ala Leu Asn Cys Asn Asp Ser  
 420 425 430  
 Leu Asn Thr Gly Trp Leu Ala Gly Leu Phe Tyr His His Lys Phe Asn  
 435 440 445  
 Ser Ser Gly Cys Pro Glu Arg Leu Ala Ser Cys Arg Pro Leu Thr Asp  
 450 455 460  
 Phe Asp Gln Gly Trp Gly Pro Ile Ser Tyr Ala Asn Gly Ser Gly Pro  
 465 470 475 480  
 Asp Gln Arg Pro Tyr Cys Trp His Tyr Pro Pro Lys Pro Cys Gly Ile  
 485 490 495  
 Val Pro Ala Lys Ser Val Cys Gly Pro Val Tyr Cys Phe Thr Pro Ser  
 500 505 510  
 Pro Val Val Val Gly Thr Thr Asp Arg Ser Gly Ala Pro Thr Tyr Ser  
 515 520 525

FIGURE 9-3

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Trp Gly Glu Asn Asp Thr Asp Val Phe Val Leu Asn Asn Thr Arg Pro  
 530 535  
 Pro Leu Gly Asn Trp Phe Gly Cys Thr Trp Met Asn Ser Thr Gly Phe  
 545 550 555 560  
 Thr Lys Val Cys Gly Ala Pro Pro Cys Val Ile Gly Gly Ala Gly Asn  
 565 570 575  
 Asn Thr Leu His Cys Pro Thr Asp Cys Phe Arg Lys His Pro Asp Ala  
 580 585 590  
 Thr Tyr Ser Arg Cys Gly Ser Gly Pro Trp Ile Thr Pro Arg Cys Leu  
 595 600 605  
 Val Asp Tyr Pro Tyr Arg Leu Trp His Tyr Pro Cys Thr Ile Asn Tyr  
 610 615 620  
 Thr Ile Phe Lys Ile Arg Met Tyr Val Gly Val Glu His Arg Leu  
 625 630 635 640  
 Glu Ala Ala Cys Asn Trp Thr Arg Gly Glu Arg Cys Asp Leu Glu Asp  
 645 650 655  
 Arg Asp Arg Ser Glu Leu Ser Pro Leu Leu Leu Thr Thr Gln Trp  
 660 665 670  
 Gln Val Leu Pro Cys Ser Phe Thr Thr Leu Pro Ala Leu Ser Thr Gly  
 675 680 685  
 Leu Ile His Leu His Gln Asn Ile Val Asp Val Gln Tyr Leu Tyr Gly  
 690 695 700

FIGURE 9-4

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Val Gly Ser Ser Ile Ala Ser Trp Ala Ile Lys Trp Glu Tyr Val Val 720  
 705 710  
 Leu Leu Phe Leu Leu Ala Asp Ala Arg Val Cys Ser Cys Leu Trp 735  
 725  
 Met Met Leu Leu Ile Ser Gln Ala Glu Ala Ala Leu Glu Asn Leu Val 750  
 740  
 Ile Leu Asn Ala Ala Ser Leu Ala Gly Thr His Gly Leu Val Ser Phe 765  
 755 760  
 Leu Val Phe Phe Cys Phe Ala Trp Tyr Leu Lys Gly Lys Trp Val Pro 780  
 770 775  
 Gly Ala Val Tyr Thr Phe Tyr Gly Met Trp Pro Leu Leu Leu Leu 800  
 785 790  
 Leu Ala Leu Pro Gln Arg Ala Tyr Ala Leu Asp Thr Glu Val Ala Ala 815  
 805  
 Ser Cys Gly Gly Val Val Leu Val Gly Leu Met Ala Leu Thr Leu Ser 830  
 820  
 Pro Tyr Tyr Lys Arg Tyr Ile Ser Trp Cys Leu Trp Trp Leu Gln Tyr 845  
 835 840  
 Phe Leu Thr Arg Val Glu Ala Gln Leu His Val Trp Ile Pro Pro Leu 860  
 850 855  
 Asn Val Arg Gly Gly Arg Asp Ala Val Ile Leu Leu Met Cys Ala Val 880  
 865 870

FIGURE 9-5

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His Pro Thr Leu Val Phe Asp Ile Thr Lys Leu Leu Leu Ala Val Phe 895  
 Gly Pro Leu Trp Ile Leu Gln Ala Ser Leu Leu Lys Val Pro Tyr Phe 910  
 Val Arg Val Gln Gly Leu Leu Arg Phe Cys Ala Leu Ala Arg Lys Met 925  
 Ile Gly Gly His Tyr Val Gln Met Val Ile Ile Lys Leu Gly Ala Leu 940  
 Thr Gly Thr Tyr Val Tyr Asn His Leu Thr Pro Leu Arg Asp Trp Ala 960  
 His Asn Gly Leu Arg Asp Leu Ala Val Ala Val Glu Pro Val Val Phe 975  
 Ser Gln Met Glu Thr Lys Leu Ile Thr Trp Gly Ala Asp Thr Ala Ala 990  
 Cys Gly Asp Ile Ile Asn Gly Leu Pro Val Ser Ala Arg Arg Gly Arg 1005  
 Glu Ile Leu Leu Gly Pro Ala Asp Gly Met Val Ser Lys Gly Trp Arg 1020  
 Leu Leu Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu 1040  
 Gly Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu 1055

FIGURE 9-6

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Gly Glu Val Gln Ile Val Ser Thr Ala Ala Gln Thr Phe Leu Ala Thr  
 1060 1065 1070  
 Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg  
 1075 1080 1085  
 Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val  
 1090 1095 1100  
 Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser Leu  
 1105 1110 1115 1120  
 Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr Arg His  
 1125 1130 1135  
 Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg Gly Ser Leu  
 1140 1145 1150  
 Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser Ser Gly Gly Pro  
 1155 1160 1165  
 Leu Leu Cys Pro Ala Gly His Ala Val Gly Ile Phe Arg Ala Ala Val  
 1170 1175 1180  
 Cys Thr Arg Gly Val Ala Lys Ala Val Asp Phe Ile Pro Val Glu Asn  
 1185 1190 1195 1200  
 Leu Glu Thr Thr Met Arg Ser Pro Val Phe Thr Asp Asn Ser Ser Pro  
 1205 1210 1215  
 Pro Val Val Pro Gln Ser Phe Gln Val Ala His Leu His Ala Pro Thr  
 1220 1225 1230

FIGURE 9-7

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Gly Ser Gly Lys Ser Thr Lys Val Pro Ala Ala Tyr Ala Ala Gln Gly  
 1235 1240 1245  
 Tyr Lys Val Leu Val Leu Asn Pro Ser Val Ala Ala Thr Leu Gly Phe  
 1250 1255 1260  
 Gly Ala Tyr Met Ser Lys Ala His Gly Ile Asp Pro Asn Ile Arg Thr  
 1265 1270 1275 1280  
 Gly Val Arg Thr Ile Thr Thr Gly Ser Pro Ile Thr Tyr Ser Thr Tyr  
 1285 1290 1295  
 Gly Lys Phe Leu Ala Asp Gly Gly Cys Ser Gly Gly Ala Tyr Asp Ile  
 1300 1305 1310  
 Ile Ile Cys Asp Glu Cys His Ser Thr Asp Ala Thr Ser Ile Leu Gly  
 1315 1320 1325  
 Ile Gly Thr Val Leu Asp Gln Ala Glu Thr Ala Gly Ala Arg Leu Val  
 1330 1335 1340  
 Val Leu Ala Thr Ala Thr Pro Pro Gly Ser Val Thr Val Pro His Pro  
 1345 1350 1355 1360  
 Asn Ile Glu Glu Val Ala Leu Ser Thr Thr Gly Glu Ile Pro Phe Tyr  
 1365 1370 1375  
 Gly Lys Ala Ile Pro Leu Glu Val Ile Lys Gly Gly Arg His Leu Ile  
 1380 1385 1390  
 Phe Cys His Ser Lys Lys Lys Cys Asp Glu Leu Ala Ala Lys Leu Val  
 1395 1400 1405

FIGURE 9-8

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Ala Leu Gly Ile Asn Ala Val Ala Tyr Tyr Arg Gly Leu Asp Val Ser  
 1410 1415 1420  
 Val Ile Pro Thr Ser Gly Asp Val Val Val Ala Thr Asp Ala Leu  
 1425 1430 1435 1440  
 Met Thr Gly Tyr Thr Gly Asp Phe Asp Ser Val Ile Asp Cys Asn Thr  
 1445 1450 1455  
 Cys Val Thr Gln Thr Val Asp Phe Ser Leu Asp Pro Thr Phe Thr Ile  
 1460 1465 1470  
 Glu Thr Ile Thr Leu Pro Gln Asp Ala Val Ser Arg Thr Gln Arg Arg  
 1475 1480 1485  
 Gly Arg Thr Gly Arg Gly Lys Pro Gly Ile Tyr Arg Phe Val Ala Pro  
 1490 1495 1500  
 Gly Glu Arg Pro Ser Gly Met Phe Asp Ser Ser Val Leu Cys Glu Cys  
 1505 1510 1515 1520  
 Tyr Asp Ala Gly Cys Ala Trp Tyr Glu Leu Thr Pro Ala Glu Thr Thr  
 1525 1530 1535  
 Val Arg Leu Arg Ala Tyr Met Asn Thr Pro Gly Leu Pro Val Cys Gln  
 1540 1545 1550  
 Asp His Leu Glu Phe Trp Glu Gly Val Phe Thr Gly Leu Thr His Ile  
 1555 1560 1565  
 Asp Ala His Phe Leu Ser Gln Thr Lys Gln Ser Gly Glu Asn Leu Pro  
 1570 1575 1580

FIGURE 9-9

SUBSTITUTE SHEET

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Tyr Leu Val Ala Tyr Gln Ala Thr Val Cys Ala Arg Ala Gln Ala Pro  
 1585 1590 1595 1600  
 Pro Pro Ser Trp Asp Gln Met Trp Lys Cys Leu Ile Arg Leu Lys Pro  
 1605 1610 1615  
 Thr Leu His Gly Pro Thr Pro Leu Leu Tyr Arg Leu Gly Ala Val Gln  
 1620 1625 1630  
 Asn Glu Ile Thr Leu Thr His Pro Val Thr Lys Tyr Ile Met Thr Cys  
 1635 1640 1645  
 Met Ser Ala Asp Leu Glu Val Val Thr Ser Thr Trp Val Leu Val Gly  
 1650 1655 1660  
 Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu Ser Thr Gly Cys Val  
 1665 1670 1675 1680  
 Val Ile Val Gly Arg Val Val Leu Ser Gly Lys Pro Ala Ile Ile Pro  
 1685 1690 1695  
 Asp Arg Glu Val Leu Tyr Arg Glu Phe Asp Glu Met Glu Glu Cys Ser  
 1700 1705 1710  
 Gln His Leu Pro Tyr Ile Glu Gln Gly Met Met Leu Ala Glu Gln Phe  
 1715 1720 1725  
 Lys Gln Lys Ala Leu Gly Leu Leu Gln Thr Ala Ser Arg Gln Ala Glu  
 1730 1735 1740  
 Val Ile Ala Pro Ala Val Gln Thr Asn Trp Gln Lys Leu Glu Thr Phe  
 1745 1750 1755 1760

FIGURE 9-10



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Trp Ala Lys His Met Trp Asn Phe Ile Ser Gly Ile Gln Tyr Leu Ala  
 1765 1770 1775  
 Gly Leu Ser Thr Leu Pro Gly Asn Pro Ala Ile Ala Ser Leu Met Ala  
 1780 1785 1790  
 Phe Thr Ala Ala Val Thr Ser Pro Leu Thr Thr Ser Gln Thr Leu Leu  
 1795 1800 1805  
 Phe Asn Ile Leu Gly Gly Trp Val Ala Ala Gln Leu Ala Ala Pro Gly  
 1810 1815 1820  
 Ala Ala Thr Ala Phe Val Gly Ala Gly Leu Ala Gly Ala Ala Ile Gly  
 1825 1830 1835 1840  
 Ser Val Gly Leu Gly Lys Val Leu Ile Asp Ile Leu Ala Gly Tyr Gly  
 1845 1850 1855  
 Ala Gly Val Ala Gly Ala Leu Val Ala Phe Lys Ile Met Ser Gly Glu  
 1860 1865 1870  
 Val Pro Ser Thr Glu Asp Leu Val Asn Leu Leu Pro Ala Ile Leu Ser  
 1875 1880 1885  
 Pro Gly Ala Leu Val Val Gly Val Val Cys Ala Ala Ile Leu Arg Arg  
 1890 1900  
 His Val Gly Pro Gly Glu Gly Ala Val Gln Trp Met Asn Arg Leu Ile  
 1905 1910 1915 1920  
 Ala Phe Ala Ser Arg Gly Asn His Val Ser Pro Thr His Tyr Val Pro  
 1925 1930 1935

FIGURE 9-11

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Glu Ser Asp Ala Ala Arg Val Thr Ala Ile Leu Ser Ser Leu Thr  
 1940  
 Val Thr Gln Leu Leu Arg Arg Leu His Gln Trp Ile Ser Ser Glu Cys  
 1955  
 Thr Thr Pro Cys Ser Gly Ser Trp Leu Arg Asp Ile Trp Asp Trp Ile  
 1970  
 Cys Glu Val Leu Ser Asp Phe Lys Thr Trp Leu Lys Ala Lys Leu Met  
 1985  
 Pro Gln Leu Pro Gly Ile Pro Phe Val Ser Cys Gln Arg Gly Tyr Lys  
 2005  
 Gly Val Trp Arg Val Asp Gly Ile Met His Thr Arg Cys His Cys Gly  
 2020  
 Ala Glu Ile Thr Gly His Val Lys Asn Gly Thr Met Arg Ile Val Gly  
 2035  
 Pro Arg Thr Cys Arg Asn Met Trp Ser Gly Thr Phe Pro Ile Asn Ala  
 2050  
 Tyr Thr Thr Gly Pro Cys Thr Pro Leu Pro Ala Pro Asn Tyr Thr Phe  
 2065  
 Ala Leu Trp Arg Val Ser Ala Glu Glu Tyr Val Glu Ile Arg Gln Val  
 2085  
 Gly Asp Phe His Tyr Val Thr Gly Met Thr Thr Asp Asn Leu Lys Cys  
 2100

FIGURE 9-12

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Pro Cys Gln Val Pro Ser Pro Glu Phe Phe Thr Glu Leu Asp Gly Val  
 2115 2120 2125  
 Arg Leu His Arg Phe Ala Pro Pro Cys Lys Pro Leu Leu Arg Glu Glu  
 2130 2135 2140  
 Val Ser Phe Arg Val Gly Leu His Glu Tyr Pro Val Gly Ser Gln Leu  
 2145 2150 2155 2160  
 Pro Cys Glu Pro Glu Pro Asp Val Ala Val Leu Thr Ser Met Leu Thr  
 2165 2170 2175  
 Asp Pro Ser His Ile Thr Ala Glu Ala Ala Gly Arg Arg Leu Ala Arg  
 2180 2185 2190  
 Gly Ser Pro Pro Ser Val Ala Ser Ser Ser Ala Ser Gln Leu Ser Ala  
 2195 2200 2205  
 Pro Ser Leu Lys Ala Thr Cys Thr Ala Asn His Asp Ser Pro Asp Ala  
 2210 2215 2220  
 Glu Leu Ile Glu Ala Asn Leu Leu Trp Arg Gln Glu Met Gly Gly Asn  
 2225 2230 2235 2240  
 Ile Thr Arg Val Glu Ser Glu Asn Lys Val Val Ile Leu Asp Ser Phe  
 2245 2250 2255  
 Asp Pro Leu Val Ala Glu Glu Asp Glu Arg Glu Ile Ser Val Pro Ala  
 2260 2265 2270  
 Glu Ile Leu Arg Lys Ser Arg Arg Phe Ala Gln Ala Leu Pro Val Trp  
 2275 2280 2285

FIGURE 9-13

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Ala Arg Pro Asp Tyr Asn Pro Pro Leu Val Glu Thr Trp Lys Lys Pro  
 2290 2295 2300  
 Asp Tyr Glu Pro Pro Val Val His Gly Cys Pro Leu Pro Pro Lys  
 2305 2310 2315 2320  
 Ser Pro Pro Val Pro Pro Pro Arg Lys Lys Arg Thr Val Val Leu Thr  
 2325 2330 2335  
 Glu Ser Thr Leu Ser Thr Ala Leu Ala Glu Leu Ala Thr Arg Ser Phe  
 2340 2345 2350  
 Gly Ser Ser Ser Thr Ser Gly Ile Thr Gly Asp Asn Thr Thr Thr Ser  
 2355 2360 2365  
 Ser Glu Pro Ala Pro Ser Gly Cys Pro Pro Asp Ser Asp Ala Glu Ser  
 2370 2375 2380  
 Tyr Ser Ser Met Pro Pro Leu Glu Gly Glu Pro Gly Asp Pro Asp Leu  
 2385 2390 2395 2400  
 Ser Asp Gly Ser Trp Ser Thr Val Ser Ser Glu Ala Asn Ala Glu Asp  
 2405 2410 2415  
 Val Val Cys Cys Ser Met Ser Tyr Ser Trp Thr Gly Ala Leu Val Thr  
 2420 2425 2430  
 Pro Cys Ala Ala Glu Glu Gln Lys Leu Pro Ile Asn Ala Leu Ser Asn  
 2435 2440 2445  
 Ser Leu Leu Arg His His Asn Leu Val Tyr Ser Thr Thr Ser Arg Ser  
 2450 2455 2460

FIGURE 9-14

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Ala Cys Gln Arg Gln Lys Lys Val Thr Phe Asp Arg Leu Gln Val Leu 2465 2470 2480  
 Asp Ser His Tyr Gln Asp Val Leu Lys Glu Val Lys Ala Ala Ala Ser 2485 2490 2495  
 Lys Val Lys Ala Asn Leu Leu Ser Val Glu Glu Ala Cys Ser Leu Thr 2500 2505 2510  
 Pro Pro His Ser Ala Lys Ser Lys Phe Gly Tyr Gly Ala Lys Asp Val 2515 2520 2525  
 Arg Cys His Ala Arg Lys Ala Val Thr His Ile Asn Ser Val Trp Lys 2530 2535 2540  
 Asp Leu Leu Glu Asp Asn Val Thr Pro Ile Asp Thr Thr Ile Met Ala 2545 2550 2555 2560  
 Lys Asn Glu Val Phe Cys Val Gln Pro Glu Lys Gly Arg Lys Pro 2565 2570 2575  
 Ala Arg Leu Ile Val Phe Pro Asp Leu Gly Val Arg Val Cys Glu Lys 2580 2585 2590  
 Met Ala Leu Tyr Asp Val Val Thr Lys Leu Pro Leu Ala Val Met Gly 2595 2600 2605  
 Ser Ser Tyr Gly Phe Gln Tyr Ser Pro Gly Gln Arg Val Glu Phe Leu 2610 2615 2620  
 Val Gln Ala Trp Lys Ser Lys Lys Thr Pro Met Gly Phe Ser Tyr Asp 2625 2630 2635 2640

FIGURE 9-15

SUBSTITUTE SHEET

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Thr Arg Cys Phe Asp Ser Thr Val Thr Glu Ser Asp Ile Arg Thr Glu  
 2645 2650 2655  
 Glu Ala Ile Tyr Gln Cys Cys Asp Leu Asp Pro Gln Ala Arg Val Ala  
 2660 2665 2670  
 Ile Lys Ser Leu Thr Glu Arg Leu Tyr Val Gly Pro Leu Thr Asn  
 2675 2680 2685  
 Ser Arg Gly Glu Asn Cys Gly Tyr Arg Arg Cys Arg Ala Ser Gly Val  
 2690 2695 2700  
 Leu Thr Thr Ser Cys Gly Asn Thr Leu Thr Cys Tyr Ile Lys Ala Arg  
 2705 2710 2715 2720  
 Ala Ala Cys Arg Ala Ala Gly Leu Gln Asp Cys Thr Met Leu Val Cys  
 2725 2730 2735  
 Gly Asp Asp Leu Val Val Ile Cys Glu Ser Ala Gly Val Gln Glu Asp  
 2740 2745 2750  
 Ala Ala Ser Leu Arg Ala Phe Thr Glu Ala Met Thr Arg Tyr Ser Ala  
 2755 2760 2765  
 Pro Pro Gly Asp Pro Pro Gln Pro Glu Tyr Asp Leu Glu Leu Ile Thr  
 2770 2775 2780  
 Ser Cys Ser Ser Asn Val Ser Val Ala His Asp Gly Ala Gly Lys Arg  
 2785 2790 2795 2800  
 Val Tyr Tyr Leu Thr Arg Asp Pro Thr Thr Pro Leu Ala Arg Ala Ala  
 2805 2810 2815

FIGURE 9-16

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Trp Glu Thr Ala Arg His Thr Pro Val Asn Ser Trp Leu Gly Asn Ile  
 2820 2825 2830  
 Ile Met Phe Ala Pro Thr Leu Trp Ala Arg Met Ile Leu Met Thr His  
 2835 2840 2845  
 Phe Phe Ser Val Leu Ile Ala Arg Asp Gln Leu Glu Gln Ala Leu Asp  
 2850 2855 2860  
 Cys Glu Ile Tyr Gly Ala Cys Tyr Ser Ile Glu Pro Leu Asp Leu Pro  
 2865 2870 2875 2880  
 Pro Ile Ile Gln Arg Leu His Gly Leu Ser Ala Phe Ser Leu His Ser  
 2885 2890 2895  
 Tyr Ser Pro Gly Glu Ile Asn Arg Val Ala Ala Cys Leu Arg Lys Leu  
 2900 2905 2910  
 Gly Val Pro Pro Leu Arg Ala Trp Arg His Arg Ala Arg Ser Val Arg  
 2915 2920 2925  
 Ala Arg Leu Leu Ala Arg Gly Gly Arg Ala Ala Ile Cys Gly Lys Tyr  
 2930 2935 2940  
 Leu Phe Asn Trp Ala Val Arg Thr Lys Leu Lys Leu Thr Pro Ile Ala  
 2945 2950 2955 2960  
 Ala Ala Gly Gln Leu Asp Leu Ser Gly Trp Phe Thr Ala Gly Tyr Ser  
 2965 2970 2975  
 Gly Gly Asp Ile Tyr His Ser Val Ser His Ala Arg Pro Arg Trp Ile  
 2980 2985 2990

FIGURE 9-17

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Trp Phe Cys Leu Leu Leu Ala Ala Gly Val Gly Ile Tyr Leu Leu  
2995 3000 3005

Pro Asn Arg  
3010

FIGURE 9-18



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/07683

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : Please See Extra Sheet.

US CL : 530/324, 389.4; 424/89; 435/5, 69.3, 252.3; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/324, 389.4; 424/89; 435/5, 69.3, 252.3; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, MEDLINE, APS, WPI

search terms: hepatitis C virus, E2, NS1, variable, antibod?, DNA, assay

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Virology, Volume 180, issued February 1991, A. J. Weiner et al., "Variable and Hypervariable Domains are Found in the Regions of HCV Corresponding to the Flavivirus Envelope and NS1 Proteins and the Pestivirus Envelope Glycoproteins", pp. 842-848, see entire document.	1-21
Y,P	Proceedings of the National Academy of Sciences U.S.A., Volume 89, issued April 1992, A. J. Weiner et al., "Evidence for Immune Selection of Hepatitis C Virus (HCV) Putative Envelope Glycoprotein Variants: Potential Role in Chronic HCV Infections", pp. 3468-3472, see entire document.	1-21
Y,P	Journal of General Virology, Volume 72, issued November 1991, H. Okamoto et al., "Nucleotide Sequence of the Genomic RNA of Hepatitis C Virus Isolated from a Human Carrier: Comparison with Reported Isolates for Conserved and Divergent Regions", pp. 2697-2704, see entire document.	1-21

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 DECEMBER 1992	Date of mailing of the international search report 02 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer D. BARND Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/07683

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

[illegible]

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/07683

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07K 5/00, 13/00; A61K 39/12; C12Q 1/70; C07H 15/12; C12N 1/20; C12P 21/00

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